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03/00020

10/501127



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Patentanmeldung Nr. Patent application No. Demande de brevet n°

02075089.9

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**Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation**

Anmeldung Nr.:
Application no.:
Demande n°:

02075089.9

Anmeldetag:
Date of filing:
Date de dépôt:

11/01/02

Anmelder:
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Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:

Diagnostics and vaccines for *Mycobacterium paratuberculosis* infections

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat:
State:
Pays:

Tag:
Date:
Date:

Aktenzeichen:
File no.
Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification Internationale des brevets:

G01N33/569, C12N5/10, C07K14/35, C12N15/31, C07K16/12, C12Q1/68, C12Q1/02, A61K39/04

Am Anmeldetag benannte Vertragsstaaten:
Contracting states designated at date of filing:
Etats contractants désignés lors du dépôt:

AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE/TR

Bemerkungen:
Remarks:
Remarques:

SEE FOR THE ORIGINAL TITLE OF THE APPLICATION ,PAGE 1 OF THE DESCRIPTION.

11. 01. 2002

Title: Paramycobacterial diagnostics and vaccines.

(52)

5 The invention relates to the field of diagnosis, treatment and prevention of Johne's disease.

M. avium subsp. *paratuberculosis* is the causative agent of paratuberculosis or Johne's disease, a chronic granulomatous infection leading to disease in ruminants that is currently responsible for substantial worldwide economic losses for farmers and the dairy industry. The presence of this bacterium in pasteurized milk in combination with its suspected role in the development of Crohn's disease has raised concern regarding its potential health effects on the human population as well. Increased awareness of the problem has resulted in renewed urgency for the development of effective diagnostics and vaccines for control and eradication of paratuberculosis.

Mycobacterium avium comprises a large group of mycobacteria that can be divided into three subspecies, *M. avium* subspecies *avium*, *M. avium* subspecies *silvaticum* and *M. avium* subspecies *paratuberculosis*. *M. avium* subspecies *avium* is widely distributed in the natural environment including soil and apparently healthy animals, as well as in birds and humans. *M. avium* subspecies *avium* isolates are opportunistic pathogens and generally cause infection and disease in immunocompromised hosts. The complete genomic sequence of *M. avium* subspecies *avium* strain 104 is currently being determined. *M. avium* subspecies *silvaticum* can produce a disease that resembles paratuberculosis in deer. Although most ruminants are infected with *M. avium* subspecies *paratuberculosis* before six months of age, clinical disease generally occurs only at four or five years of age. During this period, bacteria are believed to survive inside host cells, but extracellular episodes of infection in the lumen of the gastrointestinal tract – during which the bacterium becomes detectable in faeces – do also occur (with increasing frequency at later stages of infection). Currently available (immuno-) diagnostics against *M. avium* subspecies *paratuberculosis* have a relatively poor sensitivity, especially with respect to the detection of early or latent infection, and therefore are not effective as a tool for disease control. Whole cell Mycobacterial vaccines that are to some measure thought to be effective in freeing herds from clinical disease are

available, but these vaccines essentially interfere with the immunodiagnosis of bovine tuberculosis and do not inhibit transmission of disease.

To date several antigenic components of *M. avium subsp.*

- 5 *paratuberculosis* have been identified. The antigenic molecules of *M. avium subsp. paratuberculosis* described previously comprise glycolipids and protein antigens identified with essentially monospecific early sera raised in small experimental animals. The cell wall glycolipid molecule lipoarabinomannan (LAM) was identified by its recognition by monoclonal antibodies raised against
- 10 cell filtrate released by the bacterium, and has subsequently been purified and used for the development of a serodiagnostic ELISA (Mutharia et al., Infect. Immun. 1997. 65:387-394; Jark et al., 1997. Vet. Microbiol. 57:189-198). In addition, protein antigens with molecular weight of 14 kD (Olsen et al. Clin. Diagn. Lab. Immunol. 2001.8:797-801), 18 kD (bacterioferritin; Elsaghier et al.
- 15 Clin. Exp. Immunol. 1992 90:503-508), 19 kD (AhpD; Olsen et al., Infect. Immun. 2000. 68:801-808), 24 kD (p24BCD; Elsaghier et al. Clin. Exp. Immunol. 1992 90:503-508), 30 kD(P30; Burrels et al.; Vet. Immunol. Immunopathol. 1995. 45:311-320), 34 kD (Gilot et al. J. Bact. 1993. 175:4930-4935; De Kesel et al J. Clin. Microbiol. 1993. 31: 947-954; Coetsier et al., Clin. Diagn. Lab. Immunol.
- 20 1998. 5: 446-451), 34.5 kD(Mutharia et al., Infect. Immun. 1997. 65:387-394), , a 35 kD protein (Dheenadhayalan and Chang, unpublished data), 38 kD (Elsaghier et al. Clin. Exp. Immunol. 1992 90:503-508), 44.3 kD (Mutharia et al., Infect. Immun. 1997. 65:387-394), 45 kD (AhpC; Olsen et al., Infect. Immun. 2000. 68:801-808), 65 kD (hsp65; Koets et al., Vet. Immunol. Immunopathol.
- 25 1999. 70:105-115), 70 kD (hsp70; Stevenson et al., 1991. Nucleic Acids Res. 19:4552; Koets et al., Vet. Immunol. Immunopathol. 1999. 70:105-115), and a superoxide dismutase molecule (Dheenadhayalan and Chang, unpublished data) have been identified and (partly) characterized. Only few of these have been evaluated for the development of diagnostics or vaccines (34 kD; ; Coetsier et al.,
- 30 Clin. Diagn. Lab. Immunol. 1998. 5: 446-451), and current diagnostics and vaccines are therefore still based on rather crude antigenic materials, that cannot always be used due to interference with immune diagnosis of other ruminant mycobacterial infections such as caused by *M. bovis* or *M. tuberculosis*, and generally do not inhibit transmission of disease.

The lipoarabinomannan (Mutharia et al., Infect. Immun. 1997. 65:387-394; Jark et al., 1997. Vet. Microbiol. 57:189-198) and 34 kD antigen (Gilot et al. J. Bact. 1993. 175:4930-4935; De Kesel et al J. Clin. Microbiol. 1993. 31: 947-954; Coetsier et al., Clin. Diagn. Lab. Immunol. 1998. 5: 446-451) have been described in DE19621488 and WO9216628, for use in diagnosis and vaccines. Several other molecules have been submitted for use in diagnostics, vaccines and therapeutics. These are proteins encoded on insertion sequence ISM-1 (EP0288306 and US 5225324;), the mycobacterial DAP molecule (US9523226), a 36 kD antigen (US5776692), a soluble antigen preparation (RU2118538), an extra cellular protein with an iron-reducing capacity (DE19728834), and an acylase (WO9949054).

The invention provides a method for obtaining a recombinant host cell comprising an antigenic polypeptide fragment of *M. avium subsp. paratuberculosis* and its encoding nucleic acid comprising providing a recombinant expression library of host-cells expressing *M. avium subsp. paratuberculosis* nucleic acid, followed by e.g. plating the library for plaques and immunoscreening said library and identifying said plaques with a serum obtained from a ruminant infected with *M. avium subsp. paratuberculosis* but not with *M. bovis*, said serum essentially obtained from a late stage of infection of said ruminant with said bacterium, the method further comprising selecting a host cell that expresses a fragment that is immunoreactive with said serum.

It is preferred, from both the diagnostic as well as the vaccine viewpoint, that said recombinant host cell expresses little or no other specific Mycobacterial antigens. A useful host cell is a host cell based on *E. coli*, as further explained in the detailed description herein, but other suitable hosts cells can be derived from the art as well. Especially when such a host cell is expressing little or no other Mycobacterial antigens, it can be used directly as a whole cell preparation for a vaccine or for diagnostic purposes, however, the relevant antigenic (poly)peptide or fragments can be at least partly purified from said host cell. Ease of purification is for example obtained when the relevant antigenic peptide is tagged, for example with a his-tag as further explained in the detailed description.

It is furthermore preferred that said ruminant was found to be naturally infected with *M. avium subsp. paratuberculosis*, but has no history of infection

with tuberculosis, brucellosis or leucosis, as evidenced by finding at least two *M. avium subsp. paratuberculosis* positive feces samples within an approximately two year long period before obtaining the test-serum for use in screening, and finding essentially no antibodies or other immune responses directed against agents causing tuberculosis, brucellosis or leucosis that are nor cross-reactive with paratuberculosis antigens. When such care is taken, a serum can be obtained that is very useful in immunoscreening for *M. avium subsp. paratuberculosis* antigens, being broadly reactive against relevant *M. avium subsp. paratuberculosis* peptide fragments but bearing essentially no or only little specific reactivity with tuberculosis, brucellosis or leucosis.

As said, such a serum need be essentially obtained from a late stage of infection of said ruminant with said bacterium in order to provide a serum directed to a broad repertoire of antigens of said bacterium, however, while sufficiently maintaining its specificity for the target. It is preferred that said ruminant is a cow, leading in a most preferred embodiment to a serum such as serum 3869, which was used to identify most of the sequences described in the detailed description. Said particular serum was derived at 18-12-1996 from a naturally infected cow which tested positive for the presence of *M. avium subsp. paratuberculosis* in feces samples obtained at 10.01.1995 and 29.09.1996.

In order to identify and characterize antigens in *M. avium subsp. paratuberculosis* for use in diagnostics, therapeutics and vaccines, we have constructed a genomic expression library using the lambda TripleEx expression vector according to the Clontech manual (PT3003-1) and Stratagene Gigapack^{AE} III Gold Packaging manual. Briefly, bacterial genomic DNA isolated from *M. avium subsp. paratuberculosis* strain 316F was partially digested with *Tsp509I* and fragments of average size of 2.5 kilobasepairs, obtained by sucrose gradient centrifugation, were ligated to *EcoRI*-digested, dephosphorylated lambda TripleEx arms. The packaging reaction was carried out using Gigapack III Gold Packaging Extract and host strain *E. coli* XL1Blue (Clontech (S0924)). After plating of the library, immunoscreening of approximately 10⁸ phage plaque's was carried out with 1) a positive bovine serum (designated as 3869) and 2) control monoclonal antibodies. This resulted in our hands in the selection of 125 positive lambda TripleEx recombinants. Hundred and seventeen of these 125 positive phage recombinants were successfully converted to plasmid (pTripleEx) recombinants using the protocol described in the Clontech manual (PT3003-1).

DNA sequencing of these 117 pTriplEx allowed these to be categorized into different antigen groups (designated SEQ 1-39) with each group expressing a different antigenic protein or fragment thereof. SEQ 1-16, 21-34 were based on recombinants isolated with serum 3869, SEQ 18 on recombinants isolated with monoclonal antibodies to FabG4, SEQ 19 on recombinants isolated with monoclonal antibodies to Hsp70, and SEQ 20 on recombinants isolated with monoclonal antibodies to Hsp65 and SEQ 35-39 on recombinants isolated with respective monoclonal antibodies (13.67.1A; 10.65.3B; 13.67.2A; 10.32.3B; and 10.66.4B) directed to 5 antigenic molecules of *M. avium* subsp *paratuberculosis*. Blast searches against various data bases containing mycobacterial genomic information allowed further characterization of a number of the antigenic polypeptides and their encoding genes. Except for hsp65 and hsp70 heat shock protein antigens, none of the here provided antigenic fragments have so far been identified as a for *M. avium* subsp. *paratuberculosis* relevant antigen or figure among the already known antigens discussed above for *M. avium* subsp. *paratuberculosis*.

Using a method to obtain the desired host cells as provided herein, where special attention has been given to the selection of the serum used in immunoscreening thus results in an antigenic polypeptide fragment of *M. avium* subsp. *paratuberculosis*, obtainable from a host cell according to the invention, with hitherto unknown characteristics. In particular, the invention provides an antigenic polypeptide comprising a peptide fragment essentially derived from *M. avium* subsp. *paratuberculosis* bearing essentially no functional homology to *M. bovis* and/or *M. tuberculosis*. Here provided antigenic fragments have now been identified as an antigen relevant for *M. avium* subsp. *paratuberculosis* but do not figure among already known antigens discussed above for *M. avium* subsp. *paratuberculosis*, except for the hsp70 and hsp65 heat shock protein antigens. Using a method to obtain the desired host cells as provided herein, where special attention has been given to the serum used immunoscreening thus resulting in an antigenic polypeptide fragment of *M. avium* subsp. *paratuberculosis*, obtainable from a host cell according to the invention, with hitherto unknown characteristics, no other overlap with known antigenic fragments was found.

Having identified these antigenic polypeptide fragments, the invention provides novel antigens for diagnostic and vaccinal use in the field of *M. avium* subsp. *paratuberculosis* infections. In one embodiment, the invention provides an

antibody directed against a fragment provided herein. Such antibodies can specifically be used in detecting *M. avium subsp. paratuberculosis* antigens, but are also useful as competing antibody in a serologic assay such as an ELISA, or in other methods for testing samples for detecting *M. avium subsp.*

5 *paratuberculosis* infections. In particular, the invention relates to antigenic peptides and uses thereof (SEQ 1-16, 18, 21-39; see enclosed listing below) of *M. avium subspecies paratuberculosis* that provide novel antigens for diagnostic, therapeutic and vaccinal use in the field of *M. avium subsp. paratuberculosis* infections. The antigenic peptides SEQ 9, 10, 12-14, 22-26, 29, 30, 34 and 38 have
10 no known homologues in *M. tuberculosis/M. bovis*. The antigenic peptides SEQ 3-8, 11, 15, 16, 18, 21, 27, 28, 31-37, 39 display homologies with various ORFs predicted on the *M. bovis* and/or *M. tuberculosis* genome sequence, but none of these ORFs has thus far been identified as relevant antigenic molecules in these mycobacterial species. Both groups of antigenic peptides or fragments thereof
15 therefore provide functionally specific *M. avium subsp. paratuberculosis* antigens for development of specific diagnostics, therapeutics and vaccines, that essentially do not crossreact or interfere with the immunodiagnosis of bovine tuberculosis. The antigenic peptides SEQ 1, 2, 19 and 20 are related to previously identified antigenic molecules of other mycobacteria including
20 *M. bovis/M. tuberculosis* (resp. Erp or P34/P36 antigen; MPT53; hsp70; and hsp65). However, novel specific fragments within SEQ 1, 2 have been identified that are not present in *M. bovis/M. tuberculosis* and these are also useful for said purposes.

Thirty of the antigens (SEQ 1-16, 21-34) described herein have been
25 identified using a serum sample from a cow with a late stage infection with *M. avium subsp. paratuberculosis* but with no evidence of being infected with *M. bovis*. Such late stage sera have the advantage of being directed to a broad range of *M. avium subsp. paratuberculosis* antigens that are generated during a live infection of a cow with said bacterium. Earlier identified antigens have been
30 mainly identified by using monoclonal and polyclonal antibodies generated in mice and rabbits that have been immunised with killed (fractions) of *M. avium paratuberculosis*, and these antibodies therefore identify a different repertoire of antigens. Indeed, all 30 antigens (SEQ 1-16, 21-34) identified and characterised with the serum sample of a cow as described herein represent antigens that have
35 not been described in these earlier studies. In addition, by using a monoclonal

antibody to fabG4 and 5 other antigenic molecules of *M. avium* subsp
paratuberculosis, 6 novel antigens (SEQ18, 35-39) were identified and
characterized as described herein, and by using monoclonal antibodies to the
previously described hsp65 and hsp70 antigen homologues of these antigens
5 (SEQ20 and SEQ19) were identified and characterized as described herein.
Having identified the antigenic polypeptide fragments (SEQ 1-16, 18, 21-39; see
enclosed listing below), the invention in particular provides an isolated nucleic
acid selected from the group of SEQ 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,
16, 18, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38 or 39 and
10 a polypeptide derived thereof as novel antigens for diagnostic, therapeutic and
vaccinal use in the field of *M.avium subsp. paratuberculosis* infections. In a
preferred embodiment, an isolated nucleic acid selected from the group of SEQ 9,
10, 13, 14, 22, 23, 24, 26, 29, 30, 34 or 38 is provided, bearing less than 65%
homology with *M. tuberculosis* and/or *M. bovis*. The invention provides specific
15 antigenic peptides or fragments thereof derived from *M.avium subsp.*
paratuberculosis as provided herein for the development of specific and sensitive
diagnostics that improve specific and early diagnosis of *M.avium subsp.*
paratuberculosis. In one embodiment, the invention provides an antigenic
peptide or a fragment derived thereof, or the use of a combination of such
20 antigenic peptides or derived fragments, to specifically and sensitively detect
antibodies in whole blood, serum, milk and other tissue and body fluid samples
from animals or humans infected with *M.avium subsp. paratuberculosis*. Such
peptides or derived fragments provide use for specific detection of antibodies in
multistep serological assay formats such as an ELISA, but also in single step
25 lateral flow "dipstick" formats. In combination with specific (monoclonal)
antibodies directed against *M.avium subsp. paratuberculosis* such antigenic
peptides or derived fragments also provide use in an (ELISA)-inhibition assay
format where specific competing antibodies in above samples from humans and
animals are detected. In another embodiment, the invention provides an
30 antigenic peptide or derived fragments thereof, or a combination of such peptides
and fragments, for use in cell-mediated assay formats where specific stimulation
of a cell-mediated response - such as cell proliferation or secretion of specific
cytokines such as interferon-gamma - in immune cells in or derived from whole
blood or other body and fluid samples from animals or humans infected with
35 *M.avium paratuberculosis* is detected after stimulation with said (combination

of) antigenic peptides or derived fragments in an *in vitro* cell culture system. The invention also provides use of the specific DNA sequences or fragments thereof derived from *M. avium subsp. paratuberculosis* as provided herein for the specific detection of *M. avium subsp. paratuberculosis* DNA in tissue samples infected with *M. avium subsp. paratuberculosis*. In one embodiment, oligonucleotide primers based on the specific DNA sequences or fragments herein provide design of a DNA-based amplification test such as a PCR and/or NASBA to specifically detect *M. avium subsp. paratuberculosis* DNA in tissue samples from animals and humans infected with *M. avium subsp. paratuberculosis*. In addition, specific DNA sequences or fragments thereof provide probes for the detection of *M. avium subsp. paratuberculosis* DNA in tissue samples from animals and humans infected with *M. avium subsp. paratuberculosis* by *in situ* hybridisation tests such as tests based on PNA and bDNA.

Furthermore, the invention provides use of an antigenic polypeptide or fragment thereof derived from *M. avium subsp. paratuberculosis* as provided herein for the production of a vaccine for the treatment or prevention of *M. avium subsp. paratuberculosis* infections, in particular for the development of a vaccine that does not interfere with the immunodiagnosis of bovine tuberculosis.

In one embodiment, specific antigenic peptides or fragments herein, or a combination of such peptides and fragments, or hybrid molecules composed of specific peptides and fragments herein, provide use as subunit vaccines, in particular mixed with adjuvants such as oil-based emulsions, aluminium-based formulations, saponin-based formulations, or particle-based formulations are provided. Such vaccines are typically applied by the subcutaneous, intradermal, or intramuscular route and provide protective or therapeutic immunity to *M. avium subsp. paratuberculosis* in animals and humans, as said essentially without interfering with immunodiagnosis to bovine tuberculosis. In another embodiment, purified DNA molecules carrying DNA fragments encoding the antigenic peptides or fragments herein, or such vaccines carrying DNA fragments encoding hybrid molecules composed of (a combination of) antigenic peptides or fragments herein, provide use as DNA vaccines. DNA vaccines are typically applied by the intramuscular, intradermal or intranasal route and provide protective and therapeutic immunity to *M. avium subsp. paratuberculosis*

in animals and humans, essentially without interfering with immunodiagnosis to

bovine tuberculosis. In a third embodiment, live vaccine carriers such as *Salmonella* species expressing specific antigenic peptides or fragments herein, or expressing hybrid molecules composed of (a combination of) antigenic peptides or fragments provide live vaccines. Such vaccines are typically applied by oral route and provide protective and therapeutic immunity to *M. avium subsp. paratuberculosis* in animals and humans, essentially without interfering with immunodiagnosis to bovine tuberculosis.

Figure 1. Immunoblot displaying reactivity of serum samples from cows with a late stage infection with *M. avium subsp. paratuberculosis* B854 sonicate.

Samples

Lane M. Molecular weight markers

Lane 1-9. *M. avium subsp. paratuberculosis* B854 sonicate

Serum samples :

Lane 1. Cow 't Gen	undiluted	27-01-99
2. Cow' t Gen	1: 10	27-01-99
3. Cow 284	undiluted	12-01-99
4. Cow284	1: 10	12-01-99
5. Cow744	undiluted	27-01-99
6. Cow744	1:10	27-01-99
7. Cow744	1:50/abs <i>E.coli</i>	27-01-99
8. Cow3869	undiluted	18-12-96
9. Cow3869	1:10	18-12-96

Table 1 *M. avium subsp. paratuberculosis* antigens identified from an λ TriPLEX expression library of the genome of *M. avium subsp. paratuberculosis* 316F by serum antibodies from a healthy infected cow. Several antigens are represented by more than one independent clones selected from the library. Homology to known antigens from *M. avium paratuberculosis* (MAP) and to (predicted ORFs on) DNA contigs (ctg) of (partially completed) genome sequences of *M. avium paratuberculosis* strain K10 (MAP), *M. avium avium* strain 104 (MAA), *M. bovis* strain AF 2122/97 (MBOV) and the *M. tuberculosis* strain H37RV (MTUB) genome sequence is indicated.

Ag.	Number of clones	DNA Homologous to ctg in MAP Genome \$	DNA Homologous to ctg in MAA Genome \$	Antigenic Polypeptide homology to known AG or ORF on MAP genome \$, *	Antigenic Polypeptide Homology to AG or ORF on ctg in MAA Genome\$	Antigenic Polypeptide homology to MBOV Ag or ORF on ctg of MBOV genome\$	Antigenic Polypeptide homology to MTUB Ag or ORFs predicted on MTUB genome\$
1.	35	479 (100%)	22 (90%)	ORF on ctg 479 (100%)	ORF on ctg 22 (96%)	P36/P34 Ag (35%) (on ctg272)	Rv3810/Exp/PirG (35%)
2.	17	538 (100%)	65 (99%)	ORF on ctg 538 (100%)	ORF on ctg 65 (99%)	ORF on ctg 261 (82%)	Rv Mpt53/dsbE (82%), and DsbF (\pm 50 %)
3.	1	528 (100%)	93 (99%)	ORF on ctg 528 (100%)	ORF on ctg 93 (100%)	ORF on ctg 250 (71 %)	C-term of Rv1130 (77 %)
4.	1	498 (100%)	34 (100%)	ORF on ctg 498 (100%)	ORF on ctg 34 (100%)	ORF on ctg 252 (81%)	Rv2227 (84%)
5.	1	446 (97%)	13, (63%), 314 (63%),	ORF on ctg 446 (\geq 97%)	ORF on ctg 13, (46%), on ctg 314 (49%), and on ctg 156	ORF on ctg 276 (52 %), and on ctg272 (54%)	Rv3824c/Rv3820c/Rv1182 (Pap A1, A2, A3 (52-54

[illegible]

18.	7	529 (99%)	110 (99%)	ORF on ctg 529 (≥99%)	ORF on ctg 110 (99%)	ORF on ctg 262 (83%)	Rv0242c/FabG 4 (90%);
19.	1	469 (99%)	100 (99%)	Hsp70 (100%)	ORF on ctg 100 (99%)	ORF on ctg 260 (89%)	Rv0350/Hsp70 (89%)
20.	3	448 (100%)	116 (99%)	Hsp65 (100%)	ORF on ctg 116 (99%)	ORF on ctg 283 (95%)	Rv0440/Hsp65 (95%)
21.	1	471 (100%)	21 (99%)	ORF on ctg 471 (100%)	ORF on ctg 21 (99%)	ORF on ctg 244 (95%)	Rv0352/DNAJ (95%)
22.	1	538 (99%)	100 (95%)	ORF on ctg 538 (≥99%)	ORF on ctg 100 (≥95%)	None	None
23.	1	524 (100%)	364 (100%)	ORF on ctg 524 (100%)	ORF on ctg 364 (100%)	None	None
24.	1	526 (100%)	162 (98%)	ORF on ctg 526 (100%)	ORF on ctg 162 (≥98%)	None	None
25.	1	475 (100%)	174 (98%)	ORF on ctg 475 (100%)	ORF on ctg 174 (98%)	ORF on ctg 284 (46%), and on ctg 283 (45%)	Rv2263 (27%)
26.	1	None	174 (98%)	None	ORF on ctg 174 (≥98%)	None	None
27#.	1	404 (100%)	31 (99%)	InvA (100%)	ORFs# on ctg 31 (100%)	None	#Rv2042c (69%)
28.	1	535 (100%)	95 (98%)	ORF on ctg 535 (100%)	ORF on ctg 95 (98%)	ORF on ctg 167 (66%)	Rv3463 (66%)
29.	1	527 (100%)	150 (70%)	ORF on ctg 527 (100%)	ORF on ctg 150 (≥70%)	None	None
30.	1	537 (100%)	38 (100%)	ORF on ctg 537 (100%)	ORF on ctg 38 (100%)	None	None

31.	1	539 (99%)	221 (98%)	ORF on ctg 539 (≥99%)	ORF on ctg 221 (98%)	ORF on ctg 249 (66%)	Rv1785c/cyt P450 (67%)
32.	1	478 (100%)	123 (98%)	ORF on ctg 478 (100%)	ORF on ctg 123 (100%)	ORF on ctg 247 (50%)	Rv1867 (40%)
33.	1	511 (99%)	12 (100%)	ORF on ctg 511 (≥99%)	ORF on ctg 12 (99%)	ORF on ctg 253 (74%), and ctg 282 (63%)	Rv0590 (74%)
34.	1	516 (98%)	301 (98%)	ORF on ctg 516 (≥98%)	ORF on ctg 301 (100%)	None	Rv3232 /PvdS (35 %)
35.	12	619 (99%)	137 (93%)	ORF on ctg 510 (≥99%)	ORF on ctg 148 (98%)	ORF on ctg 213 (86%)	Rv0251c/fadE3 (86%)
36.	2	241 (100%)	223 (100%)	ORF on ctg 486 (100%)	ORF on ctg 189 (100%)	ORF on ctg 276 (61%)	Rv1326c/glgB (61%)
37.	2	196 (100%)	196 (99%)	ORF on ctg 389 (100%)	ORF on ctg 917 (99%)	ORF on ctg 265 (84%)	Rv1928c (68%)
38.	2	414 (99%)	419 (99%)	ORF on ctg 531 (99%)	ORF on ctg 43 (99%)	None	None
39.	2	385 (98%)	386 (97%)	ORF on ctg 539 (98%)	ORF on ctg 18 (97%)	ORF on ctg 265 (70%)	LppE/Rv1881c (70%)

Two different ORFs - one homologous to InvA, and another homologous to Rv2042.

* derived from DNA homology

\$ None means equal or lower than 40 % at protein level, or lower than 65 % at DNA level.

Detailed description.

Obtaining of sera.

5

The invention provides a serum obtained from a ruminant infected with *M. avium subsp. paratuberculosis* but not with *M. bovis*, said serum essentially obtained from a late stage of infection of said ruminant with said bacterium, the method further comprising selecting a host cell that expresses a fragment that is immunoreactive with said serum. It is preferred that said ruminant was found to be naturally infected with *M. avium subsp. paratuberculosis*, but has no history of infection with tuberculosis, brucellosis or leucosis, as evidenced by finding at least two *M. avium subsp. paratuberculosis* positive feces samples within an approximately two year long period before obtaining the test-serum for use in screening, and by obtaining the cows from herds that are granted an officially status declaring them free of tuberculosis, brucellosis or leucosis. When such care is taken, a serum can be obtained that is useful in immunoscreening for *M. avium subsp. paratuberculosis* antigens, being broadly reactive against relevant *M. avium subsp. paratuberculosis* peptide fragments but bearing no or at least no-detectable specific reactivity with tuberculosis, brucellosis or leucosis.

As said, such serum need be essentially obtained from a late stage of infection of said ruminant with said bacterium in order to provide a serum directed to a broad repertoire of antigens of said bacterium, however while sufficiently maintaining its specificity for the target. It is preferred that said ruminant is a cow, leading to a most preferred embodiment to a serum such as serum 3869, which was used to identify most of the sequences described in the detailed description. Said particular serum was derived at 19-12-1996 from a naturally infected cow which tested positive for the presence of *M. avium subsp. paratuberculosis* in feces samples obtained at 10.10.95 and 29.09.1996. The broad repertoire of antigens recognized by said serum 3869 is typically established by their reactivity to an whole cell preparation of *M. avium subsp. paratuberculosis* in an immunoblot (see Figure). Three other serum samples obtained at a late stage of infection from three other naturally infected cows displayed a similar broad repertoire in an immunoblot (see Figure). A similarly useful serum can experimentally be obtained from an specific-pathogen-free

(SPF) cow at a late stage of infection (typically at an age of four years or older) that has been experimentally infected within the first six months after birth. Experimental infection typically occurs orally - at three times a week for a period of four weeks - by using a tube to transport feces contaminated with *M. avium* subsp. *paratuberculosis* samples directly into the stomach.

With a serum as provided herein, the invention provides a tool for identifying or isolating hosts cells using a method for obtaining a recombinant host cell comprising an antigenic polypeptide fragment of *M. avium* subsp. *paratuberculosis* and its encoding nucleic acid comprising providing a recombinant expression library of host-cells expressing *M. avium* subsp. *paratuberculosis* nucleic acid, followed by e.g. plating the library for plaques and immunoscreening said library and identifying said plaques with said serum. Also the invention provides an isolated and/or recombinant nucleic acid comprising a sequence as identified hereunder or functional fragments thereof, derived from said host cell comprising said nucleic acid and an antigenic (poly)peptide. Also, the invention provides a nucleic acid that is essentially encoding an antigenic (poly)peptide as identified hereunder, or *M. avium* subsp. *paratuberculosis* specific variants thereof, and/or an *M. avium* subsp. *paratuberculosis* specific nucleic acid that is hybridizing under stringent conditions with a nucleic acid of which the sequence is herein provided. Also the invention provides a fragment encoded by said sequence that is hybridizing under stringent conditions with a nucleic acid of which the sequence is herein provided, thereby identifying a peptide fragment essentially derived from *M. avium* subsp. *paratuberculosis* bearing essentially a functional, or at least an antigenic, difference to a *M. bovis* and/or *M. tuberculosis* antigen.

Listing of sequences.

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 1 or functional fragments thereof, a host cell comprising said nucleic acid and an antigenic (poly)peptide and a fragment encoded by SEQ 1 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 1 comprises a DNA fragment of 1175 bp derived from recombinant phage 1821. Phage 1821 (and 34 other phages) encode *M. avium subsp. paratuberculosis* antigenic polypeptide 1.

DNA fragment of 1175 bp derived from recombinant phage 1821. Phage 1821 (and 34 other phages) encode *M. avium subsp. paratuberculosis* antigenic polypeptide 1.

15 AATTGCCTCACGATTCAATATCACCCTCTAGTAATAGGATTCCCCTCGT
 ACCATCGACTGTGTGTGATTCCTGCCAGACAGCATCGGCGGGGCGCGCCG
 ACACAACACATAGTCAGATAGAGGAGACTTCCGTGCCGAACCGACGCCGA
 CGCAAGCTTTCGACAGCCATGAGCGCGGTTCGCCGCCCTGGCAGTGGCGAG
 20 TCCTTGCGCATACTTCCTTGTCTACGAATCGACGGCCGGCAACAAGGCGCC
 CGAGCACCACGAGTTCAAGCAGGCCGCGAGTGATGAGCGATCTGCCGGGGCG
 AGCTGATGGGTGCGCTGTTCGAGGGCCTGTTCGAGTTTGGGATCAACCTG
 CCCCCGGTGCCCGCCCTGAGCGGCGGCGCCACCAGCACTCCCGGTCTGGC
 CAGCCCCGGCCTGGGTAGCCCCGGCCTGGGCACGCCCCGGCCTGGGAACGC
 25 CGGGCCTGACCAATCCCGGTCTGACGAGCCCCGGTGCGACCAGTCCCGGC
 CTGACCAGTCCCGGCCTGACCAGTCCTGGTTTGACCAGCCCCGGTCTGACC
 AGCCCCGGGTGCGGCGCCGACGACGCCCCGGGCTCACCGCGCCCCGGCGCGCT
 GCCGACCACGCCGGGCGGCGGGGTTCGCCACCCCCGGCGCCGGGCTCAACC
 CCGCGCTGTCCAACCCCCGGGCTGACCAGCCCCGGCCGGGACGGCGCCGGGG
 30 CTGGGCAGCCCCGACCGTGGCGCCGAGTGAGGTGCCGATCGACTCCGGGGC
 CGGCCTGGACCCGGGCGCCGGTGGCACGTACCCGATCCTGGGCGACCCGT
 CGACCTTCGGTAACGCCTCGCCGATCGGCGGCGGTGGCACCGGTCTGGGC
 GGCGGCTCGAGCTCGGGTGGCAGCGGCGGCCTGGTCAACGACGTGATGCA

AGCCGCCAACCAGCTCGGCGCGGGTCAGGCGATCGACCTGCTCAAGGGCC
 TGGTGATGCCGGCGATCACGCAGGGCATGCACGGCGGCGCGGCCGCGGGT
 GCTTTGCCCCGGCGCGGCCGGTGTCTGCCCCGGCGCGGCCGGCGCCCTGCC
 CGGTGCGGCCGGCGCCCTGCCGGGTGCGGCGGGCGCCGCGGGTGCGTTGC
 5 CGGCGGCCGCGCGGCCGCGCCGGCACTGCCCCCGGTCTAGACCTTTTCC
 AAACCATCCACCAGACGGCACC

Antigenic polypeptide 1 encoded by SEQ 1 – length 336 amino acids

10 VPNRRRRKLSTAMSAVAALAVASPCAYFLVYESTAGNKAPEHHEFKQAAVM
 SDLPGELMGALSQGLSQFGINLPPVPALSGGATSTPGLASPLGSPGLGTPGLG
 TPGLTNPGLTSPGATSPGLTSPGLTSPGLTSPGLTSPGAAPTTPGLTAPGALPTT
 PGGGVATPGAGLNPALSNPGLTSPAGTAPGLGSPTVAPSEVPIDSGAGLDPGA
 GGTYPILGDPSTFGNASPIGGGGTGLGGGSSSGSGGLVNDVMQAAANQLGAG
 15 QAIDLLKGLVMPAITQGMHGGAAAGALPGAAGALPGAAGALPGAAGALPGA
 AGAAGALPAAAGAAPALPPV

Comments.

- 20 1. The DNA sequence was obtained by sequence analysis of pTriplEx/1821. Sequencing of the 34 other pTriplEx recombinants showed a corresponding DNA sequence encoding antigenic peptide 1.
2. Antigenic polypeptide 1 is distantly (appr. 35 % amino acid identity) related to a smaller sized protein in *M. bovis* (secreted antigen P36/P34 precursor : Bigi et al.,
- 25 Infect. Immun. 1995. 63:2581-2586), and in *M. tuberculosis* (Erp protein : Lim et al., 1995. J. Bact. 177: 59-65; Berthet et. al. 1995. Microbiology 141:2123-2129; Berthet et. al. 1998. Science 282:759-762; Patent 6,010,855; pirG protein /Rv3810; Cole et al. 1998. Nature 393:537-544; and ID-SEQ16 in patent WO 99/09186).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 2 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 2 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding
 5 such an antigenic (poly)peptide or fragment thereof.

SEQ 2 comprises a DNA fragment of 408 bp derived from phage 2221. Phage 2221 (and 16 other phages) encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 2.

10

ACCGACGACCGCCTGCAATTCACCGCGACACGCTCAGCGGCGCGCCGTTC
 AACGGCGCCAGTCTGCAGGGCAAGCCCGCCGTGCTGTGGTTCTGGACGCCG
 TGGTGCCCGTACTGCAACGCCGAGGCCCGGGCGTGAGCCGGGTGGCCGCC
 GCCAACCCGGGCGTCACCTTCGTCGGCGTCGCCGCCACTCCGAAGTCGGC
 15 GCCATGGCCAACCTTCGTCTCCAAGTACAACCTGAACCTCACCACGCTCAACG
 ACGCCGACGGCGCGATCTGGGCCCCGCTACGGCGTGCCCTGGCAGCCCGCGT
 ACGTGTTCTACCGGGCGGACGGCAGCTCCACCTTCGTCAACAACCCACCTC
 GGCGATGCCCCAGGACGAACTGGCCGCCCGGGTGGCGGCGCTGCGCTGA

20 Antigenic polypeptide 2 encoded by SEQ 2—length 135 amino acids

TDDRLQFTATTLSGAPFNGASLQGKPAVLWFWTPWCPYCNAEAPGVSRVAA
 ANPGVTFVGVAAHSEVGAMANFVSKYNLNFITLNDADGAIWARYGVPWQPA
 YVFYRADGSSTFVNNPTSAMPQDELAARVAALRstop

25

Comments.

1. The DNA sequence was obtained by sequence analysis of pTriplEx/2221. Sequencing of the 16 other pTriplEx recombinants showed a corresponding DNA sequence encoding antigenic peptide 2.
- 30 2. One region of 4 different amino acids (residues 63-66) and 21 other amino acid differences were identified in antigenic polypeptide 2 as compared to an ORF encoded by bp 43934-44342 on ctg 261 of *M. bovis* AF 2122/97 genome sequence; www.sanger.ac.uk/Projects/M_bovis) and as compared to related proteins in *M. tuberculosis* (soluble secreted MPT53 protein Wiker et al: 1991: J. Gen.

Microbiol. 137:875-884; Wiker et. al. 1999. Microbial Pathogenesis 26:207-219;
and DsbE protein/Rv2878c: Cole et al. 1998. Nature 393:537-544) . The *M.bovis*
protein was found to be immunogenic following natural and experimental
infection with *M.bovis* in cattle (Wiker et. al. 1999. Microbial Pathogenesis
26:207-219).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 3 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 3 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid
 5 essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 3 comprises a DNA fragment of 171 bp derived from recombinant phage 2821. Phage 2128 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 3

10

AATTCCACGAACTGGCCGAGGGTGTCTCGTCACCGACGCCGAGCAGCAGCGGT
 TCCTGTGCGTCCCGAGGGCCTGCCCGCATTGCCGCCGGGCGCGGCGGGTG
 AACTCAACATCGTGGTCGATCCGGCGGTGCTGGCCACCGCCCCGGCGATTC
 CGGGCGGGATCTTCTGA

15

Antigenic open reading frame encoded by SEQ 3- length 55 amino acids

FHELAEGVVTDAEQQRFLSVAEGLPALPPGAAGELNIVVDPAVLATAPAIPGG
 IFstop

20

Comments.

1. The 170 bp DNA sequence was obtained by sequence analysis of plasmid
 25 pTriplEx/2128 derived from phage 2128.
2. Twenty three amino acid differences were identified in the polypeptide3 as compared to the C-terminal 56 amino acids of an ORF encoded by bp 7275-5707 on ctg 250 of the *M. bovis* AF 2122/97 genome sequence;
www.sanger.ac.uk/Projects/M_bovis and to the C-terminal end of an ORF in
 30 *M. tuberculosis* H37Rv (conserved hypothetical protein Rv1130: Cole et al. 1998. Nature 393:537-544).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 4 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 4 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 4 comprises a DNA fragment of 153 bp derived from recombinant phage 4224. Phage 4224 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 4.

10

TTACCTCAGGGCCATGGCTATGTCTTCACCACCCGGGAACGTCCGGTGCGCT
CGGCCCCGCGGCTGGTCCGCGGCGCCGGTACGCCACGGGGTCTCGGTGGTG
CGTTCCGGCCAGCGCTACGCGATGGGCTTGATCTTTCACGACGCCGCGTAG

15 Antigenic open reading frame encoded by SEQ 4 length 50 amino acids

LPQGHGYVFTTRERPVRSAAPVRHGVSVVRSGQRYAMGLIFHDAAs
p

20

Comments.

1. The 153 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/4224 derived from phage 4224.

25 2. Nine amino acid differences were identified in the antigenic polypeptide 4 as compared to an ORF encoded by bp 34558-34726 on ctg 252 of *M. bovis* AF 2122/97 genome sequence; www.sanger.ac.uk/Projects/M_bovis), and 8 amino acid differences were identified as compared to the C-terminal 50 amino acids of the *M. tuberculosis* H37Rv hypothetical protein Rv2227 (Cole et al. 1998. Nature
30 393:537-544).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 5 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 5 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid
 5 essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 5 comprises a DNA fragment of 551 bp derived from recombinant phage 3822. Phage 3822 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 5.

10

AATTGCTCGACGAGCGACAGACTGCCCGGTTCGAATCCGTTTGCCCTGGCGG
 CGGGCGCGCGATTTCAGTGGCGGTGTGTTTGCCCTGCGCCGCTCTCGTAGAAC
 ACGAATTGACCGGCACCGAAACGTATTACGGAGTCATTCCCGTCGACATTCG
 TCGCTCTCAGGACGAACTTGCGACAACGGGTTGGTTTGTGCGGCTTTGTGCCG
 15 CTCACCGTCTCTGTGCGGGTTCCTTCAGCAGACATCGTCCGCACCGCACAGG
 CGTCCTTCGATTTCGAACAAAGACCTTGCGAACGTTCCCGCCGAGCGCGTCG
 NGGAGATGGCGCCGTGGCTGCGGATGCCTCAGCGGGGTGCTCCTTTGGTGT
 TTTTCCTCGACGCCGGCGTGCCTCCCTATCCGCTCTCGNTAATTCGCACTT
 GGACGGTGCGAATGCCAGGCTCTACCACGACGGGAGGATTCCGTCTCAGGT
 20 CGCCATCCGGGTTAATGGGCTTGAGAGCGAAACCAAGTGATCGTGTTGCT
 CCCGAACAATCCGATCGCCCGACAATTTCGTGACCCAG

antigenic open reading frame encoded by SEQ 5—length 183 amino acids

25 LLDERQTARFESVCLAAGARFSGGVFACAALVEHELTGTETYYGVIPVDIRRS
 QDELATTGWVFGFVPLTVSVAVPSADIVRTAQASFDSNKDLANVPAERVXEM
 APWLRMPQRGAPLVFFLDAGVPPLSALXNSHLDGANARLYHDGRIPSQVAIR
 VNGLESETQVIVLLPNNPIARQFVTQ

30 Comments.

1.The 551 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/3822 derived from phage 3822.

2.Four regions of 4 or more different amino acids (53-58, 75-78, 105-108, 113-116,
 35 and 133-136) and 60 other amino acid differences were identified in the antigenic

polypeptide 5 as compared to the C-terminal parts of ORFs encoded by bp 61939-62490 on ctg 276, by bp 33910-34455 on ctg 272 and by bp 26576-27115 on ctg 272 of the *M. bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis); and to the C-terminal parts of the *M. tuberculosis* H37Rv polyketide synthase associated proteins (pap) A1, A2 and A3 (Rv3824c, Rv3820c, and Rv1182; Cole et al. 1998. Nature 393:537-544). The full sequence of antigenic polypeptide 5 is still being determined.

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 6 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 6 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid
 5 essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 6 comprises a DNA fragment of 917 bp derived from recombinant phage 5124. Phage 5124 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 6.

10
 AATTGCGCGGAGTATTCGTACAGTTCGCCGGTGC GCGGGCCGCCGGGTGTC
 AGCGCCTGCAGCACC CGGTGCACGCCCAACGTGCGCGCGCTGTCGATCTGC
 GGCGCGTGCACCACTTGCACACCGCCGCGCAGTTGCGCACCGGAGAAGTTG
 ACTTTGGCGCAGTCCTTCCCGGGGTTCGTTGAACGGCAAGGCTTTTGAGGTCT
 15 CGAGCGCGATCACCACGAAGCGGTTGCCGCGGCCCTCGGCGGTACCGCGG
 CCATGTTGCCCTGCAGATCGGCCGGCAGATCCGGCCCGCTCGCCACTTTTCG
 CGCACTGGGCGGGGTGCAAGGTCAGGCCGTGCGGCAGCTTGCGGCCGGCC
 AGCAGCTTGGGGTCGATGCCGCGTTCGCCGGTGTGCTGACCTTGTAGTCG
 GGTCCGAAGCTGGGTTTCACGTCGGCGACCTTGGCGATGTCCACCTTCGCC
 20 GGGTGAGTGGCCGATGAGCAGCCGGCCAGGACGCAGACAGCGGCCACCGC
 GCGCAACAGCTTGGACATCGTGGCCAATCTACCCAAGCGGGTGGCTCAACT
 GCGCAACGTGGACACCGTTTGGCGAGCAGATCCGCGGCGAACTGCGGTGG
 CAGCACCGGAACCGCCGCGCCGGGATCGGTGGTCAGGGTGGTGAAGGCGT
 AGTAGTCGCCCAGATAGGCGATGAATGTGTAGCTGCGCGAATCGATTTTCGG
 25 TGCCCGATTTCGACCGATGAGGTGATGTCGGCCACCATGCCAGGGTTTCGA
 CGCCGTGATGTGTGAGCCGTGCGTGAGGCGGACGCGGACGGTGGTGTGC
 CCGGCGGTCTCGGACCACTGCTGGCACGCGCCGAGCAGATCCCGCGGAAAG
 TCCACGGGCTCCGGCAAAGCCACCACCACCGCTCTACGATCCCGCCGCTG
 CC

30

Antigenic open reading frame encoded by SEQ 6— length 184 amino acids.

ATRLGRLATMSKLLRAVAAVCVLAGCSSATHPAKVVDIAKVADV KPSFGPDYK
 VSDTGERGIDPKLLAGRKLDPGLTFDPAQCAKVASGPDLPADLQGNMAAVTA
 35 EGRGNRFVVIALET SKALPFNDPGKDCAKVTFSGAQLRGGVQVVDAPQIDSA

RTLGVHRVLQALTPGGPRTGELYDYSAQ

Comments.

- 5 1. The 917 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/5124 derived from phage 5124.
2. Two regions of 4 or more different amino acids (29-33, 64-68) and 50 other amino acid differences were identified in antigenic polypeptide 6 as compared to an ORF encoded by bp 5116-5656 on ctg 720 of *M. bovis* AF 2122/97 genome
- 10 sequence (www.sanger.ac.uk/Projects/M_bovis), and to the related *M. tuberculosis* H37Rv hypothetical protein Rv0999 (Cole et al. 1998. Nature 393:537-544)..

- The invention provides an isolated and/or recombinant nucleic acid comprising
- 15 SEQ 7 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 7 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.
 - 20 Three phages (6821, 6822, and 6824) were isolated that carry SEQ 7, a DNA fragment of 314 bp encoding *M. avium subsp. paratuberculosis* antigenic polypeptide 7

AATTGGGCGTTGCGGTGGGCAGCGCGGCAGTGGCATTGACCGCCGCGGCCG
 25 GTGTCGCATCCGCCGACCCCATGGACGCGATCATCAACACCACCTGCAACTA
 CGGGCAGGTGATCGCCGCGCTGAACGCGTCCGACCCGGCGGCTGCCAGCA
 GCTGAACTCGTCGCCGATGGCGCAGTCCTACATCCAGCGGTTCTTGGCCTCC
 CCGCCGGCGAAGCGTCAGCAGATGGCCCAGCAGATCCAGGGCATGCCGGCC
 GCGCAGCAGTACATCAACGACATCAACCAGGTCGCGGTACCTGTAACAACT
 30 TCTGA

Antigenic polypeptide 7 encoded by SEQ7 -length 103 amino acids

LGVAVGSAVALTAAAGVASADPMDAINTTCNYGQVIAALNASDPAAAQQLS
 35 SPMAQSYIQRFLASPPAKRQQMAQQIQGMPAAQQYINDINQVAVTCNNFstop

Comments.

- 5 1. A part of the DNA sequence was obtained by sequence analysis of 3 pTriplEx recombinants (pTriplEx/6821, pTriplEx/6822, and pTriplEx/6824) derived from the 3 phages 6821, 6822, and 6824, respectively (bp 1-180). The full 314 basepair sequence of SEQ 7 has been obtained from *M. avium subsp. paratuberculosis* B854 genomic DNA using PCR with degenerate primers
- 10 (based on the available *M. avium avium* 104 genome sequence; available through www.tigr.org) and subsequent DNA sequencing of the amplified product.
2. One region of 5 different amino acids (residues 91-95) and 37 other amino acid differences were identified in antigenic polypeptide 7 as compared to an ORF
- 15 encoded by bp 21269-20955 on ctg 232 of the *M. bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and to the related *M. tuberculosis* H37Rv hypothetical protein Rv1174c (Cole et al. 1998. Nature 393:537-544).
- 20 The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 8 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 6 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.
- 25 SEQ 8 comprises a DNA fragment of 242 bp derived from recombinant phages 6823 and 7923. Phages 6823 and 7923 encode *M. avium subsp. paratuberculosis* antigenic polypeptide 8.
- 30 AATTATGACCGTTAAAGTGTGTTCCGCCAAGGGGGTTGGGGTGTGGCGCAG
TTATCAGCCGCCGACCGTTCGCCACAATCAAGGCATGCGTGATCGCATGAC
GGCGACGCCGCGGGCCTGCAACCGGGACCGGGTCGCGCTGCAGGCCGTGC
ACTTTTTCATGGCCGACATGGAGGCCGGCATGGGCCCGTTCCTGGGCGTGC
TGCTGCAAAGCCGTGGCTGGACCACGGGCGCCATCGGC

Antigenic open reading frame encoded by SEQ8- length 30 amino acids

IMTVKVCSAKGVGVWRSYQPPDRSPQSRHAstop

5

Comments.

1. The 242 bp DNA sequence was obtained by sequence analysis of plasmids pTriplEx/6823 and pTriplEx/7923 derived from phage6823 and 7923,
10 respectively.
2. Thusfar no significant homology was found in the antigenic polypeptide 8 as compared with ORFs encoded by the *M.bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis). One region of 5 different amino acids (1-5)
15 and 10 other amino acid differences were identified in antigenic polypeptide 8 as compared the C-terminal 30 amino acids of the related *M.tuberculosis* H37Rv hypothetical protein Rv2255c (Cole et al. 1998. Nature 393:537-544).
- 20 The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 9 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 9 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.
- 25 SEQ 9 comprises a DNA fragment of 179 bp derived from recombinant phage 1221. Phage 1221 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 9.
- 30 AATTTTTCTCACTCAGTACAAATACCTATCAGCATGGAGAAACATGGAAGAGCA
ATTACAGCCAACACGTGTAGTCTTTTAAGAGTACACCAATAAATACCCATTT
GTGAAGGTTAATTTAATGCAACCCAGGCTGTTATCTGGAATAGTATATGTCG
CCAATTCAATCCATTAAGTAATT

35

Comments.

1. The 179 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/1221 derived from phage 1221.

2. The DNA sequence and the ORFs encoded by SEQ9 show no identity with the *M. bovis* AF 2122/97 genome and predicted ORF sequences (www.sanger.ac.uk/Projects/M_bovis), nor with the genome and predicted ORF sequences of *M. tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 10 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 10 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 10 comprises a DNA fragment of 561 bp derived from recombinant phage 3226. Phage 3226 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 10.

```
AATTACCGCACGTCGTTCTGAACGCTCAGGAGGCGACGCGGAGGGCCAGAGC
GTCGCTCTTGGTGATGAATATCCGAAGCGGTGTGGGGGCGGTGAGATGAA
CGGTGACAGGAATCTTCCTGAAGGCTGGAAGGTCTATATCCGCGGTGGGTC
CGTGGAAGTGAAAGCGCCGGCGTCGGGCGGGACTTCTACCGGTTATCTACT
GACATCAGTTGAGGCCCGGCGTCTCGGTACAGAGATTCTTCGGGCGTTCCGG
CCAGTGTGACGGTGACGGGTCGATTACCGCCCAGTACATGGACCCGATCCG
GTGGATCGTCTCGCTAACGGGCGCAACGTGTGGTTGAGCGTCTCACCTGC
TGAACCAGATGGTCGATACGTGCTAAACGATGTGCGAGTCAGGCGGGCTCGC
CGTGATGTTGTTGCAAGCGTCCGTGATGGTCGAGCAGCTTGACTCAGATGC
GGTGGATGGACCTGGAAGCGAACTGCTTGAGAAGGGCTTTCGGGAAATCCA
GGCCGGGACAGAGCGTCATTCAACTGAGATCCGTTGCTTGACAGACGAATT
```

Antigenic open reading frame encoded by SEQ 10- length 186 amino acids

ITARRSNAQEATRRARASLLVMNIRSGVGAVEMNGDRNLPEGWKVYIRGGSV
 EVKAPASGGTSTGYLLTSVEARRLGTEILRAFGQCDGDGSITAQYMDPIRWIV
 SLTGRNVWLSVSPAEPDGRYVLNDVESGGLAVMLLQASVMVEQLDSDAVDG
 5 PGSELLEKGFREIQAGTERHSTEIRCLTDE

Comments.

1. The 561 bp DNA sequence was obtained by sequence analysis of plasmid
 10 pTriplEx/3226 derived from phage 3226.

2. The DNA sequence and the antigenic polypeptide 10 shows no identity with
 the *M. bovis* AF 2122/97 genome and predicted ORF sequences
 (www.sanger.ac.uk/ Projects/M_bovis), nor with the genome and predicted ORF
 15 sequences of *M. tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

The invention provides an isolated and/or recombinant nucleic acid comprising
 SEQ 11 or functional fragments thereof, a host cell comprising said nucleic acid,
 20 and an antigenic (poly)peptide and a fragment encoded by SEQ 11 or fragments
 thereof as described hereunder and an isolated or recombinant nucleic acid
 essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 11 comprises a DNA fragment of 394 bp derived from recombinant phages
 25 2922, 2925 and 7924. Phages 2922, 2925 and 7924 encode *M. avium* subsp.
paratuberculosis antigenic polypeptide 11.

AATTGACGCACTGGACGGTGGTGCATCCCTCGCTGGTCGCCGCCAACCGCG
 CCCGCCTGGCAATGCTGTTGGCCACCAACTTCTTCGGGATCAACTATCCGGC
 30 CATCGCCGAAACCGAGGCCGAGTACCACGCCATGTGGGTGAACAACCTCCGC
 GGCGATGTACCGCTACGCGGCGACCTCGGCGACCGCGGTCAGGTTGCCCCG
 GTTACCGAGCCGCCTCAGGTGGCCAACCCGTCCGGGGTGAGCACCCAGGC
 CGCGATGGTGCCCGCGACGAACGCCGCTGATTCCGGCACCCAGACCGGTGT
 CGCCGGCACCCCTGCAGGCCGCTCCACCGCCTTCTTCGATCCCAACACTGGC
 35 TGTTCAAGTACTGGAGCACCTGGGGCAACCAATT

Antigenic open reading frame encoded by SEQ11- length 131 amino acids

5 LTHWTVVHPSLVAAANRRLAMLLATNFFGINYPAlAETEAeyHAMWVNNSA
 AMYRYAATSATAVRLPRFTEPPQVANPSGVSTQAAMVPATNAADSGTQTGVA
 GTLQAASTAFFDPNTGWFKYWSTWGNQF

Comments.

- 10 1. The 394 bp DNA sequence was obtained by sequence analysis of plasmids
 pTriplEx/2922, pTriplEx/2925, and pTriplEx/7924 derived from phages 2922,
 2925 and 7924 respectively.
- 15 2. Amino acid regions 1-9 and 92-131 of antigenic polypeptide 10 shows no
 significant identity with the genome and predicted ORF sequences of the *M. bovis*
 AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), nor with the
 genome and predicted ORF sequences of *M. tuberculosis* H37Rv (Cole et al. 1998.
 Nature 393:537-544). At least one region of 4 different amino acids (46-50) and
 33 amino acid differences were identified in the region comprising the amino
 acids 10-91 of antigenic polypeptide 11 as compared to a variety of ORFs encoded
 20 by ctgs of the *M. bovis* AF 2122/97 genome sequence
 (www.sanger.ac.uk/Projects/M_bovis); and to the *M. tuberculosis* H37Rv PPE
 proteins; i.p. Rv1789, Rv1808, Rv 1809, Rv2770, Rv 3136 : Cole et al. 1998.
 Nature 393:537-544). The full sequence of antigenic open reading frame encoded
 by SEQ 11 is still being determined.
- 25 3. The *M. tuberculosis* H37Rv PPE proteins consists of a large family of proteins
 of unknown function with subfamilies that carry specific proline-rich motifs
 (Cole et al. 1998. Nature 393:537-544). PPE proteins of *M. marinum* have been
 implicated as virulence factors affecting intracellular survival (Ramakrishnam et
 al., 2000. Science 288:1436-1437).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 12 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 12 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 12 comprises a DNA fragment of 188 bp derived from recombinant phage 3823. Phage 3823 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 12.

AATTCGATGGCGATCTCATGGGCGACGACGCCGCCGAAGGACCAGCCGAGG
AGGTTGTAGGGCCCGGTGGGATCAACGCCCTGGATCCGGTCGGCATAGTTT
TTCGCCATGTCGCGGATTGACGCCGGTTCGGCCTCGCCCCGCTGCAGGGAT
TGCTGAATCCCAATGATGGGGCAGCCCAGGTAATT

Antigenic open reading frame encoded by SEQ 12— length 62 amino acids

NYLGCPHIGIQSLQRGEAEPASIRDMAKNYADRIQGVDP TGPYNLLGWSFGG
VVAHEIAIE

Comments.

1. The 188 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/3823 derived from phage 3823.

2. The amino acid region 1-40 of antigenic polypeptide 12 shows no identity with the *M. bovis* AF 2122/97 genome sequence ([www.sanger.ac.uk/ Projects/M_bovis](http://www.sanger.ac.uk/Projects/M_bovis)), nor with the predicted open reading frames of *M. tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544). The amino acid region 41-62 of antigenic polypeptide 12 shows 6 amino acid differences with an ORF encoded by bp 4798-4861 on ctg 280 of *M. bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and with the related *M. tuberculosis* ORF pks13 (Rv3800c; Cole et al. 1998. Nature 393:537-544). The *M. tuberculosis* H37Rv pks13 is a probable polyketide synthase involved in cell wall biosynthesis.

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 13 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 13 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 13 comprises a DNA fragment of 57 bp derived from recombinant phage 91211. Page 91211 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 13.

AATTTCTTTGATTGCCCACTGATTTGAGCTAGGGAGGACACTGATGACGGA
GAATT

Comments

1. The 57 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/91211 derived from phage 91211.
2. The DNA sequence and the ORFS encoded by SEQ13 show no identity with the *M. bovis* AF 2122/97 genome and predicted ORF sequences (www.sanger.ac.uk/ Projects/M_bovis), nor with the genome and predicted ORF sequences of *M. tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 14 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 14 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 14 comprises a DNA fragment of 84 bp derived from recombinant phage 2123. Phage 2123 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 14.

AATTTGCTGCCCCGCCGCTCAGCGGTTCTCCGCTGGTCACCGGCCGACTAT
AACGCAGGCTCGGCAAATCGGCGGAGTGAATT

Comments.

1. The 84 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/2123 derived from phage 2123.
2. The DNA sequence and the ORFS encoded by SEQ14 show no identity with the *M. bovis* AF 2122/97 genome and predicted ORF sequences (www.sanger.ac.uk/ Projects/M_bovis), nor with the genome and predicted ORF sequences of *M. tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

10

- The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 15 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 15 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

15

- SEQ 15 comprises a DNA fragment of 692 bp derived from recombinant phage 2126. Phage 2126 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 15.

20

AATTGCGGCAGCCCGCGCACCAACCGCGTGCGTGCCTCGGTGCGGGCGTCG
 GCGCCCAGCCGGATCATTTCCCTCGACGAACCGGAAGCGCGCCTCGAACACG
 TTCTCGGTGATCATCGAGGTGCCATCGGCGATCGACGCCAGGGCGATCGCC
 25 ATCGGCTGCAGGTCGGTGGGAAATCCGGGAAACGGCAGGGTGGCCACGTTA
 ACCGCCTTGGGCCGCTCGTACTGGGTCAACCGGAAGCTGTCGTGCGGTCTGG
 GTCACGGTGGCCCCGGCATCGTGCAACTTGTGCAGCACCACTGCAGGTGG
 GCGGGGTGACGCGCGGTACCGAGATGTCCCCGCGGGTCATCGCCGCGGCG
 ATGCCCCAGGTGCGGGCCACGATGCGGTGCGCGATCACCCGATGTTGCGGTG
 30 GGGTACAGCCGCGGCACGCCGGTGATGGTCATGGTCGGCGAACCCGCGCCC
 TCGACCTGCGCGCCCATCTGGTTGAGCATCGTGACAGATCCACCACGTGG
 GGTTGCGGGCCGCGTTGTGAATGGTGGTGACCCCTCGGCCACCACCGCC
 GGCATCAGGATGTTCTCGGTGCGCCCCACCGACGGGAACTCCAGCTGAATC
 TCCGCGCCGCGCAACGTATCCGCTGCGCCACCACGCATCCGTGCTTGATGTT
 35 TGCAGGTGGCGCCCAACTTGGCGCAAGC

Antigenic open reading frame encoded by SEQ15 – length 230 amino acids

5 ACAKLGATCKHQARMRGGAADTLRGAEIQLEFPSVGATENILMPAVVAEGVT
 TIHNAAREPDVVDLCTMLNQMGAGVEGAGSPTMTITGVPRLYPTEHRVIGDR
 IVAATWGIAAAMTRGDISVTGVDP AHLQVVLHKLHDAGATVTQTDDSFRTQ
 YERP KAVNVATLPFPGFPTDLQPMALASIADGTSMITENVFEARFRFVEEMI
 RLGADARTDGHHA VVRGLPQ

10 Comments.

1. The 692 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/2126 derived from phage 2126.
2. The amino acid region 1-25 of antigenic polypeptide 15 shows no identity with ORFs of the *M.bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), nor with the predicted open reading frames of *M.tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544)). The amino acid region 26-230 of antigenic polypeptide 15 displays 6 amino acid differences with an ORF encoded by bp 5397-6077 on ctg 157 of the *M.bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and with the *M.tuberculosis* H37Rv MurA protein (Rv1315; Cole et al. 1998. Nature 393:537-544). The *M.tuberculosis* H37Rv MurA protein is a UDP-N-Acetylglucosamine 1-carbovinyl transferase, an enzyme that functions in the cell wall peptidoglycan biosynthesis.

25

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 16 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 16 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

30

SEQ 16 comprises a DNA fragment of 420 bp derived from recombinant phage 3827. Phage 3827 encodes *M. avium* subsp. *paratuberculosis* antigenic polypeptide 16.

5 AATTGCGTGACACGCACGCCTGGTACACCTCGCCGGCGCGGATGGCTTCCA
 GGCAGGCCAGTACCCCGTCGCGGTGCGCGGCCCGGTGCGCGACGTCCCAGT
 CGATCCGGGCACGGGCGCGGCGGGCCGGCGGGCGCGGCCAGCGCGCCGGCG
 AGCCACCGCGGCATCGGCGCGCCGGTGAGGCTCTCGTACCACCACTGCCCCG
 10 TCGCGGTGCGGGCGCAGGACGCAGTCCGTCCAGCCGCGCGGCCTCGGG
 AATCCGGTTTCGGCTTGCGCTCGGCGCCGCGGTCCGGATAGGACAGGTAACC
 GATCCACCCGCCGCCACATGCGGCGGTGCGGTGTCCGCGCCGTGCCGAC
 CGCGAACACGTGCGCCGGGGGGCACC GGACGCACCGGCAGGCTCGGCGCGA
 TCACCGTCAGGGTGA

15 Antigenic open reading frame encoded by SEQ16 – length 139 amino acids

TLTVIAPSLPVRPVPPGDVFAVGTARTPQPPHVGGGWIGYLSYFDGGADAKPN
 RIPEAAGGWTDCVLRDRDQGWYESLTGAPMPRWLAGALAAPAGPPRPCR
 20 IDWDVADRAAHRDGVLACLEAIRAGEVYQACVSRN

Comments

1. The 420 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/3827 derived from phage 3827.
2. Two different regions of more than 4 amino acids (9-12, and 25-32) and 28 other amino acids differences were identified in antigenic polypeptide 16 as compared to an ORF encoded by bp 2428-2850 on ctg 194 of the *M. bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and to the related *M. tuberculosis* H37Rv pabB protein (Rv1005c; Cole et al. 1998. Nature 393:537-544). The *M. tuberculosis* H37Rv pabB protein is a probable p-aminobenzoate synthase. The full sequence of the antigenic open reading frame predicted by SEQ16 is still being determined.

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 18 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 18 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 18 comprises a DNA fragment of 1365 bp encoding antigenic peptide 18. Seven phages were isolated that carry 1323 bp of this DNA fragment encoding *M. avium subsp. paratuberculosis* antigenic polypeptide 18.

10 GTGGCTCCGAAGGTCTCGTCCGATCTGTTCTCGCAGATTGTCAATTCGGGTC
CTGGATCGTTTCTCGCCAAGCAGCTCGGCGTCCCGCAACCCGAGACGCTGC
GCCGCTACCGGCCCGGTGACCCGCCGCTGGCCGGGTGCTGCTGATCGGCG
GCGAGGGCCGCGTGGTCGAGCCGCTGCCGGCGCGCTGGCCAAGGACTAC
15 GACCTGGTCGGCAACAACCTGGGCGGGCGCTGGGCCGACCGGTTCTGGCGG
GCTGGTCTTCGACGCCACCGGGATCACCACCCCGAGGGCCTGAAGGGGCT
GTACGAGTTCTTCACCCCACTGCTGCGCAACCTGGGTCACTGCGCCCGCGTG
GTGGTGGTCGGCACCACGCCGACGCCGCCGCCGCCGACGAGCGGATC
GCCAGCGCGCCCTGGAGGGCTTCACCCGGTCATTGGGCAAGGAGCTGCGC
20 AACGGCTCGACGGTGGCGCTGGTGTACCTGTGCGCCGCCGCCAAACCCGCC
GCGACGGGCCTGGAGTCGACCATGCGGTTTCATCCTGTGCGCCAAGTCCGCC
TACGTCGACGGCCAGGTCTTCTACGTGCGCGAGGCCGACTCCACCCCCCG
GCGGACTGGGAACGGCCCGCTGGACGGCAAGGTGCGCCATCGTGACCGGTGC
GGCCCGCGGAATCGGCGCCACGATCGCCGAGGTGTTGCCCCGCGACGGCGC
25 CCGCGTGGTCGCGATCGACGTGGAATCGGCCGCCGAGACGCTGGCCGAGAC
GGCCAGCCGGGTGCGCGGCACCGCGCTGTGGCTCGACGTCACCGCCCCCGA
CGCCGTCGACAAGATCACCGAGCACCTGCGCGAGCACACGGCGGTACGCG
CGACATCCTGGTCAACAACGCCGGGATCACCCGCGACAAGCTGCTGGCCAA
CATGGACGACGCGCGCTGGGACGCCGTGTTGGCCGTGAATCTGCTTGCCCC
30 ACTTCGCCTTACCGAAGGGCTGGTGGGCAACGGCAGCATCGGCGAAGGCGG
CCGCATCGTCGGCCCTTTCTGTCGATGGCCGGCATCGCGGGCAACCGCGGCCA
GACCAACTACGCCACCACCAAGGCAGGCATGATCGGCCTCACCCAGGCGCT
GGCGCCGGAGCTCTACGACAAGGGCATCACCATCAACGCCGTGCGCGCCGG
ATTCATCGAGACCCAGATGACGGCCGCCATCCCGCTGGCCACCCGCGAGGT
35 GGGGCGCCGGATGAACTCGCTGCTGCAGGGCGGGCAGCCGGTGGACGTGC

CCGAAACCATCGCCTACTTCGCCAGCCCGGCGTCGAACGCGGTGACCGGCA
ACGTCATCCGGGTCTGCGGCCAGGCGATGCTGGGGGCATGA

Antigenic polypeptide 18 encoded by SEQ 18 – length 454 amino acids

5

VAPKVSSDLFSQIVNSGPGSFLAKQLGVPQPETLRRYRPGDPPLAGSLLIGGE
GRVVEPLRAALAKDYDLVGNLGGRWADRFGLVFDATGITTPEGLKGLYEF
FTPLLRLNLGHCARVVVVGTTPDAAAGPHERIAQRALEGFTRSLGKELRNGST
VALVYLSPAAPKPAATGLESTMRFILSAKSAYVDGQVFYVGEADSTPPADWERP
10 LDGKVAIVTGAARGIGATIAEVFARDGARVVAIDVESAAETLAETASRVGGTAL
WLDVTAPDAVDKITEHLREHHGGHADILVNNAGITRDKLLANMDDARWDAV
LAVNLLAPLRLTEGLVGNGSIGEGGRIVGLSSMAGIAGNRGQTNATTKAGMI
GLTQALAPELYDKGITINAVAPGFJETQMTAAIPLATREVGRRMNSLLQGGQP
VDVAETIAYFASPASNAVVTGNVIRVCGQAMLGAstop

15

Comments.

1. A part of the DNA sequence was obtained by sequence analysis of 7 pTriplEx recombinants derived from the 7 phages, respectively (bp 43-1365). The full
20 basepair sequence of SEQ 18 has been obtained from *M. avium subsp. paratuberculosis* B854 genomic DNA using PCR with degenerate primers (based on the available *M. avium avium* 104 genome sequence; available through www.tigr.org) and subsequent DNA sequencing of the amplified product.
2. Forty six different amino acids were identified in the antigenic polypeptide
25 18 as compared to an ORF encoded by bp 11096-12457 on ctg 262 of the *M. bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and as compared to the related *M. tuberculosis* H37Rv FabG4 protein (Rv0242c; Cole et al. 1998. Nature 393:537-544). The *M. tuberculosis* H37Rv FabG4 protein is a probable 3-OXOACYL-[ACYL-CARRIER PROTEIN] REDUCTASE.

30

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 19 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 19 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid
 5 essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 19 comprises a DNA fragment of 1872 bp encoding antigenic polypeptide 19. One recombinant phage 10.12.1A was isolated that carries 1043 bp of this
 10 fragment encoding *M. avium subsp. paratuberculosis* antigenic polypeptide 19.

ATGGCTCGTGCGGTTCGGTATCGACCTCGGGACCACCAACTCCGTCGTCGCA
 GTCCTCGAGGGCGGTGACCCCGTCGTCGTCGCCAACTCCGAGGGCTCGCGG
 ACCACCCCGTCCATCGTCGCGTTCGCCCCGCAACGGCGAGGTGCTCGTCGGC
 15 CAGCCCGCCAAGAACCAGGCGGTGACCAACGTCGACCGCACCATCCGTTCG
 GTCAAGCGGCACATGGGCACCGACTGGTCCATCGAGATCGACGGCAAGAAA
 TACACCGCTCAGGAGATCAGCGCCCGCTGCTGATGAAGCTCAAGCGCGAC
 GCCGAGGCCTATCTGGGTGAGGACATCACCGACGCGGTTCATCACCGTACCG
 GCGTACTTCAACGACGCCCAGCGTCAGGCGACCAAGGAAGCCGGCCAGATC
 20 GCCGGCCTCAACGTGCTGCGCATCGTCAACGAGCCGACCGCGGCCGCGCTG
 GCCTACGGCCTGGACAAGGGCGAGAAGGAGCAGACCATCCTGGTCTTCGAC
 CTCGGCGGCGGCACGTTTCGACGTTTCGCTGCTCGAGATCGGCGAGGGTGTG
 GTCGAGGTCCGCGCCACCAGCGGTGACAACCAACTCGGTGGCGACGACTGG
 GACGACCGGATCGTCAACTGGCTGGTTCGACAAGTTCAAGGGCACCAGCGGC
 25 ATCGACCTGACCAAGGACAAGATGGCCATGCAGCGGCTGCGTGAGGCCGCC
 GAGAAGGCCAAGATCGAGTTGTCCAGCTCGCAGAGCACCTCGATCAACCTG
 CCCTACATCACCGTCGACGCGGACAAGAACCCGCTGTTCTCGACGAGCAG
 CTGACCCGCGCCGAATTCAGCGCATCACCCAGGATCTGCTGGACCGCACC
 CGTCAGCCGTTCAAGTCGGTGATCGCCGACGCGGCATCTCGGTGTCCGAC
 30 ATCGACCACGTGGTGCTGGTGGGTGGTTCCACCCGGATGCCCGCGGTGACC
 GACCTGGTCAAGGAACTCACCGGCGGCAAGGAGCCCAACAAGGGCGTCAAC
 CCCGACGAGGTTGTGCGGGTGGGTGCCGCCCTGCAGGCCGGTGTGCTTAAG
 GGCGAGGTGAAAGACGTTCTGCTGCTTGACGTTACGCCGCTGAGCCTGGGT
 ATCGAGACCAAGGGTGGCGTGATGACCAAGCTGATCGAACGCAACACCACC
 35 ATCCCGACCAAGCGGTCCGAGACGTTACCCACGGCCGACGACAACCAGCCG

TCGGTGCAGATCCAGGTGTATCAGGGTGAGCGCGAAATCGCCGCGCACAAAC
 AAGCTGCTCGGCTCCTTCGAGCTGACCGGAATTCGCGCGGCGCCCCGCGGC
 GTGCCGCGAGATCGAGGTCACCTTCGACATCGACGCCAACGGCATCGTGAC
 GTCACCGCCAAGGACAAGGGCACCGGTAAGGAGAACACGATCAAGATCCAG
 5 GAGGGCTCCGGCCTGTCCAAGGAGGAGATCGACCGGATGATCAAGGACGCC
 GAGGCGCACGCCGAGGAGGACCGCAAGAGGCGCGAGGAAGCCGACGTCCG
 CAACCAAGCGGAATCGCTTGTCTACCAGACGGAGAAGTTCGTCAAGGACCA
 CGCGGAGGCCGAGGGCGGCTCGAAGGTTCCCGAGGAGACGCTGTCCAAGG
 TCGACGCCGCGATCGCCGACGCCAAGACGGCCCTGGGCGGCACCGACATCA
 10 CCGCGATCAAGTCGGCGATGGAGAAGCTCGGCCAGGAGTCGCAAGCGCTGG
 GACAGGCAATCTACGAGGCCACCCAGGCCGAGTCCGCCCAGGCTGGCGGGC
 CGGACGGTGCCGCGGCCGGCGGGTCCGGATCCGCCGACGATGTTGTG
 GACGCGGAGGTGGTTCGACGATGACCGGGAGTCCAAGTGA

15 Antigenic polypeptide 19 encoded by SEQ 19 -- length 623 amino acids.

MARAVGIDLGTTNSVVAVLEGGDPVVVANSEGSRTTPSIVAFARNGEVLVGQP
 AKNQAVTNVDRTIRSVKRHMGTDWSIEIDGKKYTAQEISARVLMKLKRDAEA
 20 YLGEDITDAVITVPAYFNDAQRQATKEAGQIAGLNVLRIVNEPTAAALAYGLD
 KGEKEQTILVFDLGGGTFDVSLLIEGEGVVEVRATSGDNQLGGDDWDDRIVN
 WLVDKFKGTSGIDLTKDKMAMQRLREAAEKAKIELSSSQSTSINLPYITVDAD
 KNPLFLDEQLTRAEFQRITQDLLDRTRQPFKSVIADAGISVSDIDHVVLVGGST
 RMPAVTDLVKELTGKKEPNKGVNPDEVVAVGAALQAGVLKGEVKDVLILLDV
 25 TPLSLGIETKGGVMTKLIERNTTIPTKRSETFTTADDNQPSVQIQVYQGEREIA
 AHNKLLGSFELTGIPPAPRGVPQIEVTFDIDANGIVHVTAKDKGTGKENTIKIQ
 EGSGLSKEEIDRMIKDAEAEAEEDRKREEADVNRQAESLVYQTEKFVKDQR
 EAEGGSKVPEETLSKVDAALADAKTALGGTDITAKSAMEKLGQESQALGQAI
 YEATQAESAQAGGPDGAAAGGGSGSADDVVDAEVVDDDDRESK_{stop}

30

Comments.

1. A part of the DNA sequence was obtained by sequence analysis of
 35 pTriplEx/10.12.1A recombinants derived from the phage 10.12.1A (bp 830-1872).

The full base pair sequence of SEQ 19 has been obtained from *M. avium subsp. paratuberculosis* B854 genomic DNA using PCR with degenerate primers (based on the available *M. avium paratuberculosis* hsp70 gene sequence; Stevenson et al., 1991. Nucleic Acids Res. 19:4552) and subsequent DNA sequencing of the amplified product.

2. Two amino acid differences were identified in antigenic polypeptide 19 as compared to the *M. avium paratuberculosis* HSP70 protein (Stevenson et al., 1991. Nucleic Acids Res. 19:4552). Fourty four amino acid differences were identified in antigenic polypeptide 19 as compared to an ORF encoded by bp 23807-25567 on ctg 260 of the *M. bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and to the related *M. tuberculosis* H37Rv hsp70 protein (Rv0350; Cole et al. 1998. Nature 393:537-544).

3. Patents exist describing hsp70 molecules from other (myco)bacterial species, eg. US 5,830,475 and 5,723,296.

15

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 20 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 20 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 20 comprises a DNA fragment of 1626 bp encoding antigenic polypeptide 20. Three phages (10.20.3B, 13.53.3A.2, and 10.69.4B) were isolated that carry 1615 bp of this fragment encoding *M. avium subsp. paratuberculosis* antigenic polypeptide 20.

ATGGCCAAGACAATTGCGTACGACGAAGAGGCCCGTCGCGGCCTCGAGCGG
GGGCTCAACGCCCTCGCCGACGCGGTAAAGGTCACGTTGGGCCCAAGGGT
CGCAACGTCGTCCTGGAGAAGAAGTGGGGTGCCCCACGATCACCAACGAT
GGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCGTACGAGAAGATC
GGCGCCGAGCTGGTCAAGGAAGTCGCCAAGAAGACCGACGACGTCGCCGGT
GACGGCACGACGACGGCCACGGTGCTCGCCAGGCGTTGGTCCGCGAGGG
CCTGCGCAACGTCGCGGCCGGCGCCAACCCGCTGGGTCTCAAGCGCGGCAT
CGAGAAGGCCGTCGAGAAGGTCACCGAGACCCTGCTCAAGTCGGCCAAGGA

GGTCGAGACCAAGGACCAGATCGCTGCCACCGCGGCCATCTCCGCGGGCGA
 CCAGTCGATCGGCGACCTGATCGCCGAGGCGATGGACAAGGTCGGCAACGA
 GGGCGTCATCACCGTCGAGGAGTCCAACACCTTCGGCCTGCAGCTCGAGCT
 CACCGAGGGTATGCGGTTGACAAGGGTTACATCTCGGGCTACTTCGTAC
 5 GGACGCCGAGCGTCAGGAAGCGGTCCTCGAGGACCCGTTTCATCCTGCTGGT
 CAGCTCCAAGGTCTCGACCGTCAAGGACCTGCTGCCGCTGCTGGAGAAGGT
 CATCCAGGCCGGCAAGCCGCTGCTGATCATCGCCGAGGACGTCGAGGGCGA
 GGCCCTGTCCACCCTGGTCTCAACAAGATCCGCGGCACCTTCAAGTCGGT
 GGCCGTCAAGGCGCCCGGCTTCGGCGACCGCCGCAAGGCGATGCTTCAGGA
 10 CATGGCCATCCTCACCGGCGGCCAGGTCATCAGCGAAGAGGTCCGCCCTGTC
 GCTGGAGAGCGCCGACATCTCGCTGCTCGGTAAGGCCCGCAAGGTCGTCT
 CACCAAGGACGAGACCACCATCGTCGAGGGCGCCGGTGACTCCGACGCCAT
 CGCCGGCCGGGTGGCCAGATCCGCACCGAGATCGAGAACAGCGACTCCGA
 CTACGACCGCGAGAAGCTGCAGGAGCGGCTGGCCAAGCTGGCCGGCGGCG
 15 TGGCGGTGATCAAGGCCGGCGCCGCGACCGAGGTCGAGCTCAAGGAGCGC
 AAGCACCGCATCGAGGACGCGGTCCGCAACGCCAAGGCGGCCGTGGAGGA
 GGGCATCGTCGCCGGCGGTGGCGTGGCCCTGCTGCACGCGATCCCGGCTCT
 GGACGAGCTGAAGCTCGAGGGCGAAGAGGCGACCGGCGCCAACATCGTCC
 GGGTGGCCCTCGAGGCTCCGCTGAAGCAGATCGCCTTCAACGGTGGCCTGG
 20 AGCCCGGCGTGGTGGCCGAGAAGGTCCGCAACTCGCCCGCCGGTACCGGCC
 TCAACGCCGCCACCGGTGAGTACGAGGACCTGCTCAAGGCCGGCATTGCCG
 ACCCGGTGAAGGTCACCGCTCGGCGCTGCAGAACGCGGCGTCCATCGCGG
 GGCTGTTCTGACCACCGAGGCGGTCTGCGCCGACAAGCCGGAGAAGGCGG
 CCGTCCCGCGGGCGACCCGACCGGCGGCATGGGCGGCATGGACTTCTGA

25

Antigenic polypeptide 20 encoded by SEQ20— length 541 amino acids

MAKTIAAYDEEARRGLERGLNALADAVKVTLGPKGRNVVLEKKWGAPTITND
 GVSLAKEIELEDPYEKIGAEVLVKEVAKKTDDVAGDGTTTATVLAQALVREGLR
 30 NVAAGANPLGLKRGIEKAVEKVTETLLKSAKEVETKDQIAATAAISAGDQSIG
 DLIAEAMDKVGNEGVTVEESNTFGLQLELTEGMRFDKGYISGYFVTDAERQ
 EAVLEDPFILLVSSKVSTVKDLLP LLEKVIQAGKPLLLIAEDVEGEALSTLVN
 KIRGTFKSVAVKAPGFGDRRKAMLQDMAITGGQVISEEVGLSLESADISLLG
 KARKVVVTKDETTIVEGAGDSALAGRVAQIRTEIENSDDYDREKLQERLAK
 35 LAGGVAVIKAGAATEVELKERKHRIEDAVRNAKAAVEEGIVAGGGVALLHAIP

ALDELKLEGEETGANIVRVALEAPLKQIAFNGGLEPGVVAEKVRNSPAGTG
 LNAATGEYEDLLKAGIADPVKVTRSAQNAAASIAGLFLTTEAVVADKPEKAAA
 PAGDPTGGMGGMDFstop

5 Comments.

1. A part of the DNA sequence was obtained by sequence analysis of 3 pTriplEx recombinants (pTriplEx/10.20.3B, pTriplEx/13.53.3A.2, and pTriplEx/10.69.4B) derived from the 3 phages 10.20.3B, 13.53.3A.2, and 10.69.4B). The full 1626
 10 basepair sequence of SEQ 20 has been obtained from *M. avium subsp.*

paratuberculosis B854 genomic DNA using PCR with degenerate primers (based on the available *M. avium avium* 104 genome sequence; available through www.tigr.org) and subsequent DNA sequencing of the amplified product.

2. Seven and two amino acids differences were identified in antigenic polypeptide
 15 20 as compared hsp60 sequences of *M. avium paratuberculosis* strain Linda described by el-Zaatari et al. (Clin. Diagn. Lab. Immunol. 2: 657-664, 1995; Accession numbers AAA996679 and P42348) One region of 6 different amino acids (528-533) and 26 other amino acid differences were identified in antigenic polypeptide 20 as compared to an ORF encoded by bp 78660-77041 on ctg 283 of
 20 the *M. bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and to the related *M. tuberculosis* H37Rv hsp60 (GroEL2/Rv0440; Cole et al. 1998. Nature 393:537-544)

25 The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 21 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 21 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

30

SEQ 21 comprises a DNA fragment of 364 bp derived from recombinant phage 8921. Phage 8921 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 21.

35

AATTCAAGGAGATCAGCGTCGCCTACGAGGTGCTCAGCGACCCGGAGAAAC
 GGCGGATCGTCAACCTGGGCGGTGATCCGCTGGAGGGGGCGCCGCGGCG
 GGCGGCGGCTTCGGAGGGTTCGGCGGCCTCGGCGATGTGTTCGAGGCCTTT
 TTCGGGGGCGGCTTCGGCGGCGGCACCACTCCCGCGGCCCGATCGGGCG
 5 GGTCCGGCCGGGCTCGGATTCGCTGCTGCGGATGCGGCTCGACCTCGAGGA
 GTGCGCCACCGGGGTGACCAAGCAGGTCACCGTCGACACCGCCGTGCTGTG
 CGACCGCTGCCACGGCAAGGGCACCAACGGCGACTCCGCCCCGGTGCCATG
 CGACACCTG

10 The antigenic open reading frame encoded by SEQ 21; length 120 amino acids

FKEISVAYEVLSDPEKRRIVNLGGDPLEGAAAAGGGFGGFGGLGDVFEAFFG
 GGFGGGTTSRGPIGRVVRPGSDSLLRMRLDLEECATGVTKQVTVDTAVLCDRC
 HGKGTNGDSAPVPCDT

15

Comments.

1. The 364 bp DNA sequence was obtained by sequence analysis of plasmid
 pTriplEx/8921 derived from phage 8921.

20 2. Ten different amino acids were identified in antigenic polypeptide 21 as
 compared to an ORF encoded by 19565-19924 on ctg 244 of the *M. bovis* AF
 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and to the
 related *M. tuberculosis* H37Rv DNAJ protein (Rv0352; Cole et al. 1998. Nature
 393:537-544). DNAJ acts with GrpE to stimulate DnaK ATPase (hsp70) activity.

25

The invention provides an isolated and/or recombinant nucleic acid comprising
 SEQ 22 or functional fragments thereof, a host cell comprising said nucleic acid,
 30 and an antigenic (poly)peptide and a fragment encoded by SEQ 22 or fragments
 thereof as described hereunder and an isolated or recombinant nucleic acid
 essentially encoding such an antigenic (poly)peptide or fragment thereof.

DNA fragment of 240 bp derived from recombinant phage 5524. Phage 5524
 35 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 22.

AATTCAACGAGATTCTCGCGAGGGGATCCGCACCTTGGCGGTTCAAGTATC
 AGCAATCGTCCGCAGCAGAGTTAGCGGCAATTCATGGCAACCATCAGAGCC
 AACGTTCTCAACACACCTTGGTTTACCAAACGTCCAGATAGGTTCTGTCAGCA
 5 TCGAGAAGGTCAACACGTTCTGAGCCNCGGATCAGGTTGCCGCGCAACGGG
 ATTCGTCAGCGGCCGAAGTCCGACGGCCGGAATT

Comments.

10

1. The 240 bp DNA sequence was obtained by sequence analysis of plasmids pTriplEx/5524.2 and pTriplex/5524.3 derived from phage 5524.

2. The DNA sequence and the ORFs encoded by SEQ22 show no identity with the *M. bovis* AF 2122/97 genome and predicted ORF sequences

15 (www.sanger.ac.uk/ Projects/M_bovis), nor with the genome and predicted ORF sequences of *M. tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

20 The invention provides an isolated and/or recombinant nucleic acid comprising
 SEQ 23 or functional fragments thereof, a host cell comprising said nucleic acid,
 and an antigenic (poly)peptide and a fragment encoded by SEQ 23 or fragments
 thereof as described hereunder and an isolated or recombinant nucleic acid
 essentially encoding such an antigenic (poly)peptide or fragment thereof.

25

SEQ 23 comprises a DNA fragment of 61 bp derived from recombinant phage 4223. Phage 4223 encodes *M. avium* subsp. *paratuberculosis* antigenic polypeptide 23.

30 AATTCGGTGATGAAGGCGGCGCGCTGCGCCACCGGGATCTCCTGCAACTGC
 TTGAGTAATT

Antigenic open reading frame encoded by IDSEQ 23 – length 19 amino acids

35 LLKQLQEIPVAQRAAFTE

Comments.

- 5 1. The 61 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/4223 derived from phage 4223.
2. The DNA sequence and the antigenic polypeptide 23 show no identity with the *M.bovis* AF 2122/97 genome and predicted ORF sequences (www.sanger.ac.uk/Projects/M_bovis), nor with the genome and predicted ORF sequences of
- 10 *M.tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

- The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 24 or functional fragments thereof, a host cell comprising said nucleic acid,
- 15 and an antigenic (poly)peptide and a fragment encoded by SEQ 24 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof:

- SEQ 24 comprises a DNA fragment of 94 bp from recombinant phage 7926.
- 20 Phage 7926 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 24.

AATTACGTCACTGTGACGCCGCGATCGGTGCGGGCCCGAATCCGCCCGGCC
GGTGCCGGGTGGCTCGGCGAAATCGCATGTGCACCAACAAATT

- 25 Comments.

1. The 94 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/7926 derived from phage 7926.
2. The DNA sequence and the ORFS encoded by SEQ24 show no identity with
- 30 the *M.bovis* AF 2122/97 genome and predicted ORF sequences (www.sanger.ac.uk/Projects/M_bovis), nor with the genome and predicted ORF sequences of *M.tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 25 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 25 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid
 5 essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 25 comprises a DNA fragment of 83 bp derived from recombinant phage101022. Phage 101022 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 25 .

10

AATTGGCCGGCCAAGCGGGCCGGGACCGCCGCGCTGTGGGAGCTGTCC
 GAGGAACTGACCGGGACCAAGTTTCCGCTCTGA

Antigenic open reading frame encoded by SEQ 25 – length 27 amino acids

15 NWPAKRAGTAAALWELSEELTGTKFPL_{stop}

Comments.

1. The 83 bp DNA sequence was obtained by sequence analysis of plasmid
 20 pTriplEx/101022 derived from phage 101022.
2. The antigenic polypeptide 25 shows no identity with ORFS predicted on the *M. bovis* AF 2122/97 genome sequence ([www.sanger.ac.uk/Projects/M bovis](http://www.sanger.ac.uk/Projects/M_bovis)). The amino acid region 1-12 of antigenic polypeptide25 shows no identity with the genome and predicted open reading frames of *M. tuberculosis* H37Rv (Cole et al.
 25 1998. Nature 393:537-544). Eight amino acid differences were identified in the amino acid region 13-27 of antigenic polypeptide 25 as compared to the C-terminal 17 amino acids of a related *M. tuberculosis* possible oxidoreductase protein (Rv2263; Cole et al. 1998. Nature 393:537-544), and to the C-terminal 16 amino acids of *M. tuberculosis* proteins Rv0068 and Rv0439c (Cole et al. 1998.
 30 Nature 393:537-544).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 26 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 26 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof. . .

SEQ 26 comprises a DNA fragment of 59 bp derived from recombinant phage 5921. Phage 5921 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 26.

AATTCCAACGGCGCGTGTGCCNCGGCGCCCGCNCNGACTNCCTATCGGNGA
ACGCAATT

Comments.

15

1. The 59 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/5921 derived from phage 5921.

2. The DNA sequence and the ORFs encoded by SEQ26 show no identity with the *M. bovis* AF 2122/97 genome and predicted ORF sequences

(www.sanger.ac.uk/ Projects/M_bovis), nor with the genome and predicted ORF sequences of *M. tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 27 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 27 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 27 comprises a DNA fragment of 374 bp derived from recombinant phage 6923. Phage 6923 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 27.

AATTGGCCGGGGCCGCCGGCCTCGTCGTCGGGCTGCTGGGGTTGCCGGCGC
TGGGAATCGCCGCGGCCGGGCTGGTGGTGTCTTCGTCGGTGCGGTGC

TGACGCACCTGCGGGCCGGCGTGCTGTACAACATCGCGTTTCCCGGGGCCT
 ACCTGTGTCTGTCCGCGGCATCGCTGGCCTGGATGGCTCTGCGCTGAATCG
 GGCGCGAGGTCAGGCCGGGAAGTAGCGGATCCGGTTGATCGCGTTGGCCCG
 CCAGGCCACATCGGCGAACATGATGGCGCGGCCGTGACCCGAATGCAGCGA
 5 GACCGCCACGGCGGGGCCGGCGCTGTTTCATCTTGGTGAGGCCCGCCCCCGC
 CAACTGTTTCGGCCAATT

antigenic open reading frame 1 encoded by SEQ 27-- length 65 amino acids

10 LAGAAGLVVGLLGLPALGIAAAAGLVVFFVGAVLTHLRAGVLYNIAFPAYLC
 LSAASLAWMALRstop

Antigenic open reading frame 2 encoded by SEQ 27-- length 52 amino acids

15 LAEQLAGAGLTKMNSAGPAVAVSLHSGHGRAIMFADVAWRANAINRIRYFPA
 stop

Comments.

- 20 1. The 374 bp DNA sequence was obtained by sequence analysis of plasmid
 pTriplEx/6923 derived from phage 6923.
2. The antigenic open reading frame 1 of SEQ27 is identical to the
M.paratuberculosis InvA protein (Bull et al., Microbiology 2000. 146:2185-2197).
 No significant homology was identified between the antigenic open reading
 25 frame 1 encoded by SEQ 27 as compared to ORFs encoded by the *M.bovis* AF
 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), nor with the
 predicted open reading frames of *M.tuberculosis* H37Rv (Cole et al. 1998. Nature
 393:537-544). Sixteen different amino acids were identified in antigenic open
 reading frame 2 of SEQ27 as compared to an ORF encoded by bp 16729-16884 on
 30 ctg 246 of the *M.bovis* AF 2122/97 genome sequence
 (www.sanger.ac.uk/Projects/M_bovis), and to the related open reading frame
 Rv2042c of *M.tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 28 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 28 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid
 5 essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 28 comprises a DNA fragment of 215 bp derived from recombinant phage 7922. Phage 7922 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 26.

10

AATTACGTGCACAACCTGGCTGCGGCTGGGGTTCACCGAGGCCGACGTGCGC
 CCACCGGGCAGCGACCGGCTGATCGACGCGGTGATCAGCTACGGCACCCCG
 CAGGCCGTCGCGGCGCGACTGCGCGAGCATCTGGACGCCGGGGCCGACCA
 CGTGGCGATCCAGGTGCTGGGCGGGGATTCCGAGGAGACGCTGCTGCCCGC
 15 GCTGACCGAATT

Antigenic open reading frame encoded by SEQ 28 – length 71 amino acids

NYVHNWLRRLGFTEADV RPPGSDRLDAVISYGTPQAVAARLREHL DAGADHV
 20 AIQVLGGDSEETLLPALTE

Comments.

1. The 215 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/7922 derived from phage 7922.
- 25 2. One region of 4 different amino acids (61-64) and 26 other amino acid differences were identified in antigenic polypeptide 28 as compared to an ORF encoded by bp 2940-3146 on ctg 167 of the *M. bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and to a related *M. tuberculosis* H37Rv ORF (the probable neuraminidase Rv3463; Cole et al. 1998. Nature 393:537-544).

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The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 29 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 29 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid
 5 essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 29 comprises a DNA fragment of 206 bp derived from recombinant phage 2121. Phage 2121 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 29.

10

AATTATCGTCCTTCGGCTCGTCCTCGCAGTACGCCATTCCCGAACCAGTGCC
 CGCATAGCGGGTTCCGTTCGGCGTTGGTTCCCGGGGGCAGGGCATCGACGGT
 CTGCTGGATGTAGTGGATCACTGTCTTCTGCGCTTCTCCTTGACTCGCAGGT
 ATTTTCGGAGACGGGGCGGCTGGTGTGCGATTCCATCGGACCTCCTGGAATT

15

Comments.

1. The 206 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/2121 derived from phage 2121.
- 20 2. The DNA sequence and the ORFS encoded by SEQ29 show no identity with the *M. bovis* AF 2122/97 genome and predicted ORF sequences (www.sanger.ac.uk/ Projects/M_bovis), nor with the genome and predicted ORF sequences of *M. tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

25

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 30 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 30 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid
 30 essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 30 comprises a DNA fragment of 175 bp derived from recombinant phage 4925. Phage 4925 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 30.

35

AATTCCTCGGCGACGATGGCCACTTCGACACCCGAGGCGCCGTCGGAGCGC
 AACGACCGCCAGCCGGACATCTCGATCTGCTTGTCAGCCGGCTGATTCGG
 CCCTGGTTCGTCGAGATCCTGCACCGTTGCCGGGCCGAGGTCGTCCGCCAGC
 TTGGCGGCGGCATCACGCAATT

5

Comments.

1. The 175 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/4925 derived from phage 4925.
- 10 2. The DNA sequence and the ORFs encoded by SEQ30 show no identity with the *M. bovis* AF 2122/97 genome and predicted ORF sequences (www.sanger.ac.uk/Projects/M_bovis), nor with the genome and predicted ORF sequences of *M. tuberculosis* H37Rv (Cole et al. 1998: Nature 393:537-544).

15

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 31 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 31 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid

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essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ31 comprises a DNA fragment of 755 bp derived from recombinant phage 6924. Phage 6924 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 31.

25

- AATTCGTGTTGGGTTGCCGCGCAGGCAATATGGACGTGCACCCCGACAGAT
 GACCGGAGATCCGAGCGCATGAGCGAAGGCCAATACGGCACCTTCCACCTA
 CCCC GGCTGGATTTTTCGAACGTTGCCGATGAGCGTCGATCGCGGTCTGGG
 GTGGAAGACGTTGCGCGACGCCGACCGGTGGTGTTCATGAACGGCCACTA
 30 CTACCTCACCCGCCGGGAGGATGTGCTGGCGGCGCTGCGCAACCCGAAGGT
 GTTCTCGTCGACGGTGCTGCAACCTCCCGGGCATCCGCTGCCGGTGCTGCC
 GTTGGCATTTGACCCGCCGCGCAGCACACCCGCTATCGCAAAATCCTGCAGCCG
 TATTTAGCCCGCACGCGCTGGGCAAGTCCCGGCCGGTGCTGGAGCGCCAT
 GCCGCAGAGATGATCGCCGCGTTGGCCGACCGTGGCGAGTGCGAAGTGATG
 35 GCGGATTTTCGCGCACCTGTATCCGTTCCAGGTGTTTCATGGACCTCTACGGCC

TGCCGCTGCAGGATCGCGACCGCCTGCTCGACTGGAAAAACGCCGTCGTCG
 GCGAGAAGCCGTTTCGTCACCGAGTCCGACGTCGAGAAGTCCGAGCAACTGC
 TGGCCTATCTCGCGGACGCGATCGCCCAGCGCCGGCAGCACCCCGGCACCG
 ACATGCTGTGCGCAGGTGATGACCGGCGAGGGCAACTTCACCGACATCGAAT
 5 TGCTGGGAATGAGCCACCTGCTGATCCTGGCCGGGCTTG

Antigenic open reading frame encoded by SEQ31 – length 228 amino acids

MSEGQYGTFFHLPRLDFATLPMSVDRGLGWKTLRDAGPVVFMNGHYLTRRE
 10 DVLAALRNPKVFSSTVLQPPGHPLPVLPLAFDPPQHTRYRKILQPYFSPHALG
 KSRPVLERHAAEMIAALADRGECEVMADFAHLYPFQVFMDLYGLPLQDRDR
 LLDWKNNAVVGEPFVTSDEKSEQLLAYLADAIAQRRQHPXTDMLSXVMT
 GEGNFTDIELLGMSHLLXLAGL

15

Comments.

1. The 755 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/6924 derived from phage 6924.
- 20 2. At least 4 regions of 4 or more different amino acids (164-167, 176-180, 186-189, and 209-213) and 57 other amino acid differences were identified in the antigenic polypeptide 31 as compared to an ORF encoded by bp 16029-16715 on ctg 249 of the *M. bovis* AF 2122/97 genome sequence ([www.sanger.ac.uk/Projects/M bovis](http://www.sanger.ac.uk/Projects/M_bovis)), and to the related *M. tuberculosis* H37Rv cytochrome P450 (Rv1785c; Cole et al. 1998. Nature 393:537-544).
- 25

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 32 or functional fragments thereof, a host cell comprising said nucleic acid,
 30 and an antigenic (poly)peptide and a fragment encoded by SEQ 32 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ 32 comprises a DNA fragment of 190 bp derived from recombinant phage
 35 9121. Phage 9121 encodes *M. avium* subsp. *paratuberculosis* antigenic

polypeptide 32.

AATTGGCCTGGCCGGTCCCGCATTTCGCTGACCGTCTCGGCGATGCCGTGC
 AGCGAGTAGTTGTTGCCCGGTCCGCCGAAGTACGGCAGGCCGCCGGTGAGC
 5 GTCAGGCCGCGCGGATCGTCGGTGGCCAGGCCCGTGCCGTCCGAGAAGTTG
 AACACCGGCACCGGGAAGCAGCTGTACAGATCGAACG

Antigenic open reading frame of SEQ 32 – length 62 amino acids

10 FDLYSCFPVPVFNFCDGTGLATDDPRGLTLTGGLPYFGGPGNNYSLHGIAETV
 SEMRDRPGQ

Comments.

15 1. The 190 bp DNA sequence was obtained by sequence analysis of plasmid
 pTriplEx/9121 derived from phage 9121.
 2. Two regions of 4 or more different amino acids (12-18, and 51-59) and 17 other
 amino acid differences were identified in antigenic polypeptide32 as compared to
 an ORF encoded by bp 27852-28037 on ctg 247 of the *M.bovis* AF 2122/97
 20 genome sequence ([www.sanger.ac.uk/Projects/M bovis](http://www.sanger.ac.uk/Projects/M_bovis)), and to the related
M.tuberculosis H37Rv possible actetyl CoA synthase (Rv1867; Cole et al. 1998.
 Nature 393:537-544).

25 The invention provides an isolated and/or recombinant nucleic acid comprising
 SEQ 33 or functional fragments thereof, a host cell comprising said nucleic acid,
 and an antigenic (poly)peptide and a fragment encoded by SEQ 33 or fragments
 thereof as described hereunder and an isolated or recombinant nucleic acid
 essentially encoding such an antigenic (poly)peptide or fragment thereof.

30

SEQ33 comprises a DNA fragment of 744 bp derived from recombinant phage
 7921. Phage 7921 encodes *M. avium subsp. paratuberculosis* antigenic
 polypeptide 33.

35 AATTCGTCTGGGGCCGCCAGTACGGGGAGAACACGATCAACCCATGAGAAT

CACCGGTACCGCAGTCAAACCTCGTCGTCTTCTGGTCCGTGCTGGCGATGTTT
 ACAGTGATGATCATCGTCGTGTTTCGGCCAGGTCCGATTTCGATCGGACCACCG
 GCTACTCCGCGGTGTTACCGACGCCGGCGGGTTACGGGCCGGGCAGTTTCG
 TGCGCGCCTCCGGGGTGAGGTGGGCAAGGTCGCCGCCGTGACGCTTTCCG
 5 ACAAGGACAGCCGGGTGTTGGTGGAGTTCAACGTGGATCGCTCACTGGCAC
 TGGACCAGGGCACCACCGCGTCGATCCGCTACCTCAACCTGATCGGCGACC
 GCTATCTGGAACCTCAAGCGCGGCACCAGCGGCCGCCGGCTGCCCCCGGGTG
 GCCGCATCCCGGTGAGCACACTCAGCCGGCGTTGGATCTCGACGCGCTGA
 TCGGCGGATTCCGGCCGCTGTTCCAGGCTTTGGACCCGAACAAGGTCAACA
 10 GCATCGCCCAGTCCATCATCACCGTGTTCAGGGACAGGGCGCCACCATCAC
 CGACATCCTCGACCAGACCGCGCGCTGACCGCCGCGCTGGCCGACCGCGA
 CAAGGCGATCGGCGAGGTGATCAACAACCTGAACACCGTGCTGGCCACCAC
 CGTCAAGCACGAGAAAGAGTTTCGACCGAACGGTCGACAAGTTGGAACCTGCT
 GATCACCGGATTGAAGAACCCGGGCCG

15

Antigenic open reading frame encoded by SEQ 33 – length 247 amino acids

IRLGPPVRGEHDQPMRITGTAVKLVVFWSVLAMFTVMIVVFGQVRFDRRTGY
 SAVFTDAGGLRAGQFVRASGVEVGKVAAVTLSDKDSRVLVEFNVDRLALDQ
 20 GTTASIRYLNLIQDRYLELKRGTSGRRLPPGGRIPVEHTQPALDLDALIGGFRP
 LFQALDPNKNVNSIAQSIITVFQGGGATTTDILDQTAALTAALADRDKAIGEVN
 NLNTVLATTVKHEXEFDRTVDKLELLITGLKNPG

25 Comments.

1. The 744 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/7921 derived from phage 7921.

30

2. Forty six amino acid differences were identified in the antigenic
 polypeptide33 as compared to an ORF encoded by bp 23706-24440 on ctg253 of
 the *M.bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis),
 and a related *M.tuberculosis* H37Rv hypothetical protein (Rv0590; Cole et al.
 35 1998. Nature 393:537-544).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 34 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 34 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ34 comprises a DNA fragment of 590 bp derived from recombinant phage 6222. Phage 6222 encodes *M. avium subsp. paratuberculosis* antigenic polypeptide 34.

AATTCGGCTTCGTAGACGGCGTCGGGAATCTTCACCGCGGCCCGGGCCGAT
 TTCTTCTTGCCGGCCTTGAGGGGAACGCCGTCATTTTTCGCGCTGCTCACAG
 CCACGAATGATAGCCCCACGCAGGGATTTTCAGCGCTGTCTAGCGCTGCCTA
 15 AGACCGGGCGGCCTCCACCAGTTCCTCGAGCGCCTTGGTTCAGCAATCGGG
 GCAGCAGGTCGACGTCGCTCATCGCTTCGCGGTCGGCGNTGATGCCGTAGT
 AGAGCATGCCGNTGTAGGACGTCACGCNGATGGCCAGCGCCTGGTTGTGCA
 GCAGCGGCGGCACCGAGTAGGTCTCCAGCAGCTTGGTGCCGGCGACGTACA
 TCTGCGACTGCGCCCCGGGCGCGTGGTGATCAACAGGTTGAAGGTGCGCT
 20 TGGAGAAGCTCGGGAAGCTGGTCGCGACCCGGATGCCCATGGCGTGACGGG
 TGGGCGGGGCGAAACCCGACAGCGTGACGATGGTCCC GGCGTCCACCAGAT
 GCGCGGCCCGGGCTGGATTGCGCGGCGTGCGCGATCTGGGAGAGCCGC
 ACCACCGCGTTCGGCTCCCCACCGGCC

25 Antigenic open reading frame by SEQ 34 – length 35 amino acids

VAVSSAKNDGVPLKAGKKKSARAAVKIPDAVYEAE

30 Comments.

1. The 590 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/6222 derived from phage 6222.
2. The DNA sequence and antigenic polypeptide 34 show no identity with the
- 35 *M. bovis* AF 2122/97 genome and predicted ORF sequences (www.sanger.ac.uk/)

Projects/*M. bovis*), nor with the genome and predicted ORF sequences of *M. tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

- 5 The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 35 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 35 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

10

SEQ 35 comprises a DNA fragment of 625 bp derived from recombinant phages 4112111. Phage 4112111 (and 11 other phages) encode *M. avium subsp. paratuberculosis* antigenic polypeptide 35.

- 15 AATTCGCGCATACCCGTCCTGGTCACAACGCCACATGCTGGTAGGCTGT
GGAATCGAGGGTCAATCCGGATCGGACCCCAACGTCGACTTGTGGGCGCC
AATTCGCGGGTTTTTCGCCCAGCAAGTCGACGTTTCGGCGCGAATCGGTGAG
GTGGGCACAGGTGAATGACGAAGAGGACATGCTGGTCGCCACGGTGCGG
GCGTTCATCGACCGCGAGGTCAAACCGACCGTGCGCGAGGTGGAGCACGC
20 CGATGCCTATCCCGAGGCGTGGATCGAGCAGATGAAGCGGATCGGGATCT
ACGGGCTGGCGGTGCCCCGAGGAATACGGTGGTTCGCCGGTGTCCATGCCG
TGCTACGTGCGGGTCACCGAGCAGCTGGCGCGCGGCTGGATGAGCCTGGC
CGGGGCGATGGGCGGGCACACCGTGGTGGCCAAGCTGCTAACGCTGTTCG
GCACCGAGGACCASAAGCGGGCCTACCTGCCGCGGATGGCCAGCGGCGA
25 AATCCGGGCCACCATGGCGTTGACCGAGCCCSGCGGCGGCTCGGACCTGC
AGAACATGTCGACCACCGCGCTGCCCCGACCCCGACTCCGACGGNCTGGTG
GTCAACGGGGCCAAGACCTGNATCAAC

Antigenic open reading frame by SEQ 35 – length 117 amino acids

30

MLVATVRAFIDREVKPTVREVEHADAYPEAWIEQMKRIGIYGLAVPEEYGG
PVSMPCYVRVTEQLARGWMSLAGAMGGHTVVAKLLTLFGTEDXKRAYLPR
MASGEIRATMALTEP

Comments.

1. The 625 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/4112111 derived from phage 4112111.
2. Fifteen amino acid differences were identified in the antigenic polypeptide 35 as compared to an ORF encoded by bp 18838-19188 on ctg 213 of the *M. bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and to the related *M. tuberculosis* H37Rv FadE3 protein (Rv0251c; Cole et al. 1998. Nature 393:537-544).

The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 36 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 36 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ36 comprises a DNA fragment of 241 bp derived from recombinant phages 4422111 and 4422112. Phage 4422111 (and 4422112) encode *M. avium subsp. paratuberculosis* antigenic polypeptide 36.

GTGGGGGCAAGCCAATTACGTTTCGCATCGACCCGGCACAGGCGGTCGCTC
ACGTCATCAACATGCCGCTCATCCCCGATGAGGCTCGAATGACCTTGCTAC
GCAGGCGCTGAACGCACGACGAAACGGACCGGAGGTGAAAGGGACATGA
GCCACGCCGATCAACTCGCTCGGACGCACCTGGCGCCCGATCCTGCGGAC
CTGTGCGCGCTGGTCGCCGGCACCCACCACGACCCGCACGG

Antigenic open reading frame by SEQ 36 – length 31 amino acids

MSHADQLARTHLAPDPADLSRLVAGTHHDPH

Comments.

1. The 241 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/4422111 (and pTriplEX4422112) derived from phage 4422111 and 4422112.
2. At least 1 region of 4 or more different amino acids (3-7) and 8 other amino acid differences were identified in the antigenic polypeptide 36 as compared to a ORF encoded by bp 2559-2651 on ctg 276 of the *M. bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and to the related *M. tuberculosis* H37Rv glgB protein (Rv1326c; Cole et al. 1998. Nature 393:537-544).

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The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 37 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 37 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ37 comprises a DNA fragment of 236 bp derived from recombinant phages 4722141 and 4722142. Phages 4722141 and 4722142 encode *M. avium subsp. paratuberculosis* antigenic polypeptide 37.

GGACACCAACGTGACCGGGGTGTTTCTCACCGCCCAGGCGGCGGCCCGGG
CGATGATGCGGCAGGGCCGCGGCGGCCATCATCACCACCGCCTCGATG
TCCGGGCACATCATCAACGTCCCGCAGCAGGTCGGCCACTACTGCGCCAG
CAAGGCGGCCGTGATCCAGCTGACCAAGGCCATGGCCGTCGAATTCTGCA
GGATCCGTCGACTCTAGACTCGAGCAAGCTTATGCA

Antigenic open reading frame by SEQ 37 – length 70 amino acids

NVTGVFLTAQAAARAMMRQGRGGAIITASMSGHIINVPQQVGHYCASKAA
VIQLTKAMAVEFCRIRRLX

Comments.

1. The 236 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/4722141 (and pTriplEx/4722142) derived from phage 4722141 and
5 4722142.

2. Eight other amino acid differences were identified in the antigenic polypeptide 37 as compared to an ORF encoded by bp 1360-1545 on ctg 265 of the *M. bovis* AF 2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and to *M. tuberculosis* H37Rv ORF (Rv1928c; Cole et al. 1998. Nature 393:537-544).

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The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 38 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 38 or fragments
15 thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

SEQ38 encodes a DNA fragment of 419 bp derived from recombinant phages 4222121 and 4222122. Phages 4222121 and 4222122 encode *M. avium subsp.*
20 *paratuberculosis* antigenic polypeptide 38.

CGGCCACCGCACCCAGGGGAGGCCATGACTCACACCAAGGCCGGTCGTGC
CGCGTGCGCCGGCCGCTGCGCGGTCGTCTGTCCGCCGCCGCGCTGTTGTG
CGCGGCAGCGGCCGCCGCGGACGAAGCCGATGACGCGTTCCTCGCCGGCC
25 TGGCCAAGGGCGGGATCACCATGTTTCGACGACGACGACGCGATCGCCATG
GGCCACAGCGTGTGCTCGAGCATCGACGCCAACCCCAACGTGTTCGATGCT
GGCGCTGCGGCTGACCAAGCAAACCCCGTTGACGCCGAAGCAATCCGGCT
ACTTCATCGGTCTTTTCGGTCGCCAGCTACNTGCCCGCAGTACAAGGACGA
CGTCGACCCCTCGCTGGGCTGGCTGATCCCGCCGCCGCTGATGTGANGTTG
30 CCGGCCGGCATCGGCGT

Antigenic open reading frame by SEQ 38 – length 125 amino acids

MTHTKAGRAAWPAACAVVLSAAALLCAAAAADEADDAFLAGLAKGGITM
 FDDDDAIAMGHSVCSSIDANPNVSMMLALRLTKQTPLTPKQSGYFIGLSVASYX
 PAVQGRRRPLAGLADPAAADVX

5 Comments.

1. The 419 bp DNA sequence was obtained by sequence analysis of plasmid pTriplEx/4222121 (and pTriplEx/4222122) derived from phage 4222121 and 4222122
 2. The DNA sequence and antigenic polypeptide 38 show no identity with the *M. bovis*
- 10 AF 2122/97 genome and predicted ORF sequences (www.sanger.ac.uk/Projects/M_bovis), nor with the genome and predicted ORF sequences of *M. tuberculosis* H37Rv (Cole et al. 1998. Nature 393:537-544).

- 15 The invention provides an isolated and/or recombinant nucleic acid comprising SEQ 39 or functional fragments thereof, a host cell comprising said nucleic acid, and an antigenic (poly)peptide and a fragment encoded by SEQ 39 or fragments thereof as described hereunder and an isolated or recombinant nucleic acid essentially encoding such an antigenic (poly)peptide or fragment thereof.

20 SEQ39 comprises a DNA fragment of 392 bp derived from recombinant phages 4622122 and 4622121. Phages 4622122 and 4622121 encode *M. avium subsp. paratuberculosis* antigenic polypeptide 39.

25 CGGCGTAGCATCGTCAAGTCGTTGCCCGCGCTGATGCCGGAGCGGCAGTA
 AGGAGTTCGGCTGGTGCAAAAACGCTTGCCACAGTCGTTTTGGTGCTGA
 CGGCCGTTGTCGCCGGTATCGCCGGGTGCAGCGCGGCGCAGACCGTGCCG
 CGCAAGGCCCGCCGGCTGACCATCGACGGTGCCACCCACACGACCCGCCC
 GCCGTCCTGCCGGCAGGACCAGATGTATCGGACCATCAACATCCCCGACC
 30 ACGACGGTGGAGTCGAAGCGGTGGTGCTGCTCAGCGGTTACCGGGTGATG
 CCGCAGTGGGTGAAGATCCGGAACGTCGACGGCTTACCGGCAGTCTACT
 GGCCASGGCGGAGTGGGCGACGCGCACGTCGATCTCACMAAT

Antigenic open reading frame by SEQ 39 – length 101 amino acids

GVRLVQKRLPTVVVLVLTAVVAGIAGCSAAQTVPRKAARLTIDGATHHTTRPPSC
RQDQMYRTINIPDHDGGVEAVLLSGYRVMPQWVKIRNVDGFTGSLLA

5

Comments.

1. The 392 bp DNA sequence was obtained by sequence analysis of plasmid
pTriplEx/4622122 (and pTriplEx/4622121) derived from phage 4622122 and
10 4622121.

2. 29 amino acid differences were identified in the antigenic polypeptide 39 as
compared to an ORF encoded by bp 51316-51600 on ctg 265 of the *M. bovis* AF
2122/97 genome sequence (www.sanger.ac.uk/Projects/M_bovis), and to the related
M. tuberculosis H37Rv cytochrome c hsp70 protein (Rv1881c; Cole et al. 1998. Nature
15 393:537-544).

Examples of use.

5 The currently available validated serodiagnostic and cell-mediated immune have
 a relative good specificity in the detection of a specific immune response to *M*
avium subsp. paratuberculosis but clearly are not sensitive enough to be useful
 in detection and control of paratuberculosis. A problem that is solved here is
 therefore the development of immunodiagnostic tests for paratuberculosis with
 an improved sensitivity, particularly useful for early diagnosis of
 10 paratuberculosis, by detecting a differentiating immune response against one of
 above identified specific antigenic peptide fragments, comprising or consisting of
 a peptide fragment essentially derived from *M. avium subsp. paratuberculosis*
 bearing essentially a functional, or at least an antigenic, difference to a *M. bovis*
 and/or *M. tuberculosis* antigen. Of course, nucleic acid based differential
 15 diagnostic techniques are now also provided for differentiating diagnostic use.

The currently approved vaccines are all based on whole bacteria and display fair
 protection to clinical paratuberculosis, but do however interfere with the
 immunodiagnosis of bovine tuberculosis, still an important veterinary problem in
 20 many countries. We therefore provided here improved (subunit)vaccines that do
 not or only negligibly induce cross-immunity to bovine tuberculosis. In particular
 the genes encoding the antigenic polypeptides 1, 2, 7, 18, and fragments thereof
 are useful. To establish the usefulness of these antigens for inclusion in an
 immunodiagnostic assay and for preparation of a subunit vaccine for inducing
 25 protective efficacy, these antigenic polypeptide 1, 2, 7, 18, were over-expressed
 and purified as recombinant proteins. Hereto, we expressed the antigenic
 polypeptides as poly-histidine tagged fusion proteins in *E. coli* XL1Blue by
 cloning DNA fragments, obtained by PCR amplification using specific primers
 and genomic DNA from *M. avium paratuberculosis* strain B854, into the vectors
 30 pQE80, pQE81 and pQE82 (obtained from QIAGEN) by employing standard
 molecular biological procedures known to individuals skilled in the art. Briefly,
 the amplified DNA fragments encoding antigenic polypeptide 1, 2, 7, 18, 19 and
 20 were cloned into the vector PCR4-TOPO (Invitrogen) according to the
 manufacturers instruction manual; The inserted DNA fragments encoding
 35 antigenic polypeptide 1, 2, 7, 18 were subsequently confirmed by DNA

sequencing, and subcloned into the expression vectors PQE80-82 series.

Expression of the now his-tagged antigenic polypeptides 2, 7, 18 was visualised by Coomassie Brilliant Blue staining of SDS-PAGE, and by western blotting with serum 3869 (antigenic polypeptide 1, 2 and 7) or with the respective monoclonal antibodies reactive with antigenic polypeptide 18. The recombinant antigenic polypeptides 1, 2, 7, 18 were subsequently purified to a purity of > 95 % (as detected by Coomassie Brilliant Blue stained SDS-PAGE) by immobilised metal-chelate affinity chromatography according to the QIAGEN manual for purification ("The QIAexpressionist"; fourth edition-january 2000).

- 10 Contaminating *E. coli* lipopolysaccharide was removed to a level of < 50 EU/mg protein by affinity chromatography using Detoxi-Gel Endotoxin Removing Gel (Pierce) according to the protocol provided by the manufacturer. The thus antigenic polypeptides retained their recognition by serum 3869 (antigenic polypeptide 1, 2 and 7) or by the respective monoclonal antibodies reactive with
15 antigenic polypeptides 18 both as tested by immuno-blotting and by dot-blotting.

- The serodiagnostic relevance of the purified antigenic polypeptides 1, 2, 7, 18, is tested by evaluating particularly their sensitivity (but also their specificity) both in a direct ELISA format and in an ELISA inhibition format using specific
20 monoclonal antibodies. Hitherto we use panels of serum samples (but also tissue and body fluid samples such as for example whole blood and milk) obtained from ruminants, more specifically cows, sheep and goats, that are naturally or experimentally infected with *M. avium subsp. paratuberculosis*, and in various, particularly early, stages of infection. These antigenic polypeptides prove in
25 principle particularly sensitive (and specific) as compared to currently used and validated absorbed ELISA test but may be further investigated for the purpose of improving and validating the particular serodiagnostic format, with appropriate methods known in the art, but also by evaluating these antigenic polypeptides in a variety of other serodiagnostic formats including single step lateral flow
30 "dipstick" formats. If indicated, and to improve sensitivity and specificity also specific subfragments or deleted forms of antigenic polypeptides 1, 2, 7, 18, be produced by cloning and expression of truncated forms of the genes encoding antigenic polypeptides 1, 2, 7, 18, 19, and 20, or by introducing specific deletions into the genes encoding antigenic polypeptides 1, 2, 7, 18 by the procedures
35 described above. Again, (a combination of) these products are evaluated for

sensitivity (and specificity) in the above indicated serodiagnostic formats and compared with the validated absorbed ELISA assay.

5 The diagnostic relevance of the purified antigenic polypeptides 1, 2, 7, 18 in cell-mediated assays will be further tested by evaluating particularly their sensitivity (but also their specificity) initially in a whole blood format where secretion of interferon-gamma is detected by comparing with the validated BOVIGAM assay. We will use whole blood samples obtained from ruminants,
10 more specifically cows, sheep and goats, naturally or experimentally infected with *M. avium subsp. paratuberculosis*, in various, particularly early, stages of infection. (A combination of) those antigenic polypeptides proving particularly sensitive (and specific) as compared to currently used and validated BOVIGAM test will be further investigated by improving and validating the particular
15 format, but also by evaluating other *in vitro* cell culture formats including for example by use of cell proliferation as a read out. If indicated, and to improve sensitivity and specificity also specific subfragments or deleted forms of antigenic polypeptides 1, 2, 7, 18 can be produced by cloning and expression of truncated forms of the genes encoding antigenic polypeptides 1, 2, 7, 18 or by
20 introducing specific deletions into the genes encoding antigenic polypeptides 1, 2, 7, 18 by the procedures described above. Again, these products are evaluated for sensitivity (and specificity) in the above indicated cell-mediated assay formats and compared with the validated BOVIGAM assay.

25 To evaluate the usefulness of the purified polypeptides 1, 2, 7, 18 for developing improved vaccines that do not interfere with immunodiagnosis for bovine tuberculosis, subunit vaccines are prepared from the host cells provided herein based on the purified antigenic polypeptides mixed with strong adjuvants (an
30 oil-based emulsion is particularly useful), immunise goats and evaluate the induction of skin-test reactivity with bovine and paratuberculosis derived PPDs. (A mixture of) those subunit vaccines positive with paratuberculosis PPD, but negative with bovine PPD are evaluated for protective efficacy by comparing the protective efficacy with currently available whole cell based vaccines using the
35 goat model. In addition, (a mixture of) of (sub)vaccines based on other adjuvants

and based on DNA vaccines carrying the genes encoding the antigenic polypeptides 1, 2, 7, 18, are tested. The protective efficacy resembles or exceeds the efficacy that is achieved by a currently available whole cell vaccine but a vaccine according to the invention has the added advantage that it does not interfere with immunodiagnoses of (bovine) tuberculosis.

Claims

11.01.2002

(52)

1. A method for obtaining a recombinant host cell comprising an antigenic polypeptide fragment of *M. avium subsp. paratuberculosis* and its encoding nucleic acid comprising providing an recombinant expression library of host-cells expressing *M. avium subsp. paratuberculosis* nucleic acid and immunoscreening said library with a serum obtained from a ruminant infected with *M. avium subsp. paratuberculosis* but not with *M. bovis*, said serum essentially obtained from a late stage of infection of said ruminant with said Mycobacterium, the method further comprising selecting a host cell that expresses an antigenic peptide fragment that is immunoreactive with said serum.
2. A method according to claim 1 wherein said ruminant is a cow.
3. A host cell obtainable by a method according to anyone of claims 1 to 2.
4. An antigenic polypeptide comprising a peptide fragment essentially derived from *M. avium subsp. paratuberculosis* bearing essentially no functional homology to *M. bovis* and/or *M. tuberculosis*.
5. A peptide according to claim 4 obtainable from a host cell according to claim 3.
6. A nucleic acid encoding a peptide according to claim 4 or 5.
7. An antibody directed against a peptide according to claim 4 or 5.
8. Use of a peptide according to claim 4 or 5 and/or a nucleic acid according to claim 6 and/or an antibody according to claim 7 in a method for testing samples for detecting *M. avium subsp. paratuberculosis* infections.
9. A diagnostic test kit for the detection of *M. avium subsp. paratuberculosis* infections comprising a peptide according to claim 4 or 5 and/or a nucleic acid according to claim 6 and/or an antibody according to claim 7.
10. Use of a host cell according to claim 3 and /or a fragment according to claim 4 or 5 and/or a nucleic acid according to claim 6 for the preparation of a vaccine for the treatment or prevention of *M. avium subsp. paratuberculosis* infections.
11. A vaccine for the treatment or prevention of *M. avium subsp. paratuberculosis* infections comprising a host cell according to claim 4 and /or a fragment according to claim 4 or 5 and/or a nucleic acid according to claim 6.

11. 01. 2002

Abstract

(52)

The invention relates to the field of diagnosis, treatment and prevention of Johne's disease. The invention provides a method for obtaining a recombinant host cell comprising an antigenic polypeptide fragment of *M. avium subsp. paratuberculosis* and its encoding nucleic acid comprising providing an recombinant expression library of host-cells expressing *M. avium subsp. paratuberculosis* nucleic acid and immunoscreening said library with a serum obtained from a ruminant infected with *M. avium subsp. paratuberculosis* but not with *M. bovis*, said serum essentially obtained from a late stage of infection of said ruminant with said Mycobacterium, the method further comprising selecting a host cell that expresses a fragment that is immunoreactive with said serum.

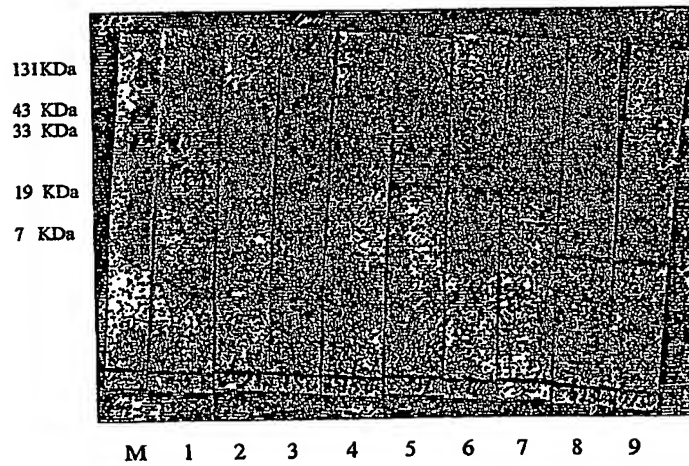
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FIGURE 1



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